Volume 24, No. 6

November-December, 1959

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THE MAIN PATH OF NITROGEN ASSIMILATION IN BAC. SUBTILIS*

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In the preceding work [1] the authors showed that cells of a normal strain of BS and many other forms of the genus Bacillus contain AD-ase (acting with participation of DPN) which catalyzes the reductive amination of PA, and that GD-ase is usually absent in these forms. It was suggested that AD-ase might be the enzyme which assured the primary act in ammonia assimilation in this group of microbes, analogous to the function of GD-ase in other organisms [1].

Wiame and co-workers [2, 3] observed that a BS mutant which could not use NH_4^+ for growth (Am^- strain) had AD-ase and lacked GD-ase; mutants which assimilated NH_4^+ (Am^+ strains) contained GD-ase. Wiame and co-workers [4-7] worked chiefly with Am^- strains and could not show the participation of AD-ase in the assimilation of ammonia in BS; the inability of these Am strains to assimilate ammonia was ascribed by them to the absence of GD-ase.

The data presented below confirm the role of AD-ase in the assimilation of NH_4^+ in BS (and probably in other forms of Bacillus which lack GD-ase) and indicate the reason for the inability of a number of normal strains of BS to grow on ammonia synthetic media which contain glucose, glycerol, or some other organic metabolite as the sole source of carbon.

MATERIALS AND METHODS

For most of the experiments we used a strain of the "wild" type, BS N-1235 [1]. For demonstrations of the conditions of growth we used a synthetic basic medium like that used before [1] but the sources of carbon or nitrogen were changed depending on the conditions of the experiment. The medium contained: $2.5 \cdot 10^{-2}$ M of the source of carbon, $2.5 \cdot 10^{-2}$ M of the source of nitrogen, $5.7 \cdot 10^{-3}$ M K_2HPO_4, $2.0 \cdot 10^{-4}$ M $MgSO_4$, $6.5 \cdot 10^{-5}$ M $FeSO_4$, $9.0 \cdot 10^{-4}$ M $CaCl_2$, $2.0 \cdot 10^{-3}$ M NaCl. The solution was sterilized by autoclaving for 20 min at a pressure of 8 kg/cm^2. When the sources of carbon were glucose or ketoacids, they were autoclaved separately and added to the medium under aseptic conditions.

Study of growth of bacteria. The growth of the bacteria was studied by seeding a standard amount of weighed cells washed from a 24 hour culture on slanting agar into 50 ml of basic medium in a 250 ml Erlenmeyer flask. The flask was incubated at 37° on a shaking machine with a rotatory motion. Samples of culture were collected at 3 hour intervals and after dilution (1:3) were measured for optical density in a spectrophotometer (at 570 mμ).

*Abbreviations in the text: BS) Bac. subtilis; AD-ase)-L-alanine dehydrogenase; GD-ase) L-glutamic acid dehydrogenase; AL) alanine; GL) glutamic acid; AS) aspartic acid; KG) α-ketoglutaric acid; PA) pyruvic acid; CAC) citric acid cycle; OA) oxaloacetic acid; DPN) diphosphopyridine nucleotide; TPN) triphosphopyridine nucleotide.

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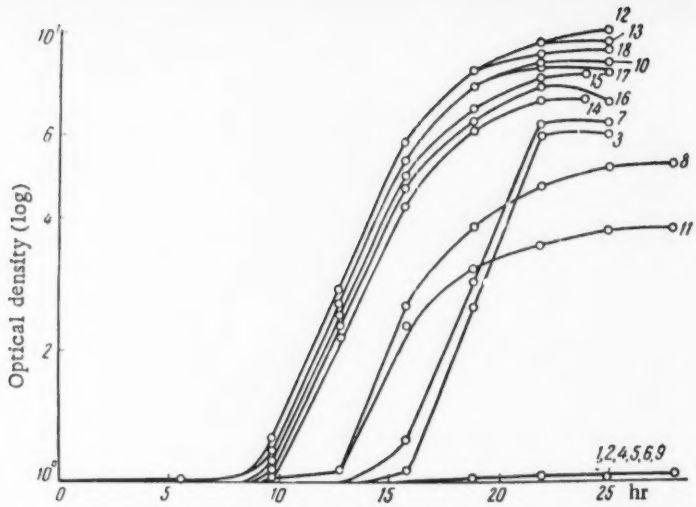


Fig. 1. Growth curve of Bac. subtilis N-1235 on a synthetic medium: 1) NH_4^+ + glucose; 2) NH_4^+ + glycerol; 3) NH_4^+ + citrate; 4) NH_4^+ + KG; 5) NH_4^+ + succinate; 6) NH_4^+ + fumarate; 7) L-AL; 8) L-Gl; 9) DL-AS; 10) NH_4^+ + citrate + glycerol; 11) NH_4^+ + KG + glycerol; 12) NH_4^+ + + succinate + glycerol; 13) NH_4^+ + fumarate + glycerol; 14) L-Al + + fumarate; 15) L-Gl + fumarate; 16) L-GL + glycerol; 17) DL-AS + + glycerol; 18) L-AL+glycerol. Growth at 35°.

Growth of Bac. subtilis N-1235 on Media with Different Sources of Carbon and Nitrogen. Basic sources of C and N taken in equimolecular amounts — $2.5 \cdot 10^{-2}$ M. When glycerol was used as a supplementary nutritive substance, its concentration was $1.5 \cdot 10^{-2}$ M

Source of C and N	Optical density after 24 hour growth	Source of C and N	Optical density after 24 hour growth
NH_4^+ + glucose	0.020	The same + succinate + + glycerol	0.960
The same + glycerol	0.024	The same + fumarate + + glycerol	0.940
The same + succinate	0.018	The same + KG + glycerol	0.360
The same + fumarate	0.015	L_{-AL} (alone)	0.635
The same + KG	0.020	L_{-GL} (alone)	0.510
The same + PA	0.580	D_{-GL} (alone)	0.082
The same + citrate	0.600	D_{-GL} + glycerol	0.582

Analytical methods. The utilization of O_2 was measured by the ordinary manometric process. Free amino acids were determined in the protein-free filtrate as described earlier [1]. Ketoacids were transformed into the 2,4-dinitrophenylhydrazones and identified qualitatively by paper chromatography; the solvent was n-butanol saturated with 3% aqueous ammonia [8]. The hydrazone spots were developed by spraying with a 2% NaOH solution.

RESULTS

To explain whether BS could utilize NH_4^+ for growth by alteration of the mechanism of transamination [9, 10] in which the first link is the synthesis of AL catalyzed by AD-ase, we studied the growth of these bacteria on

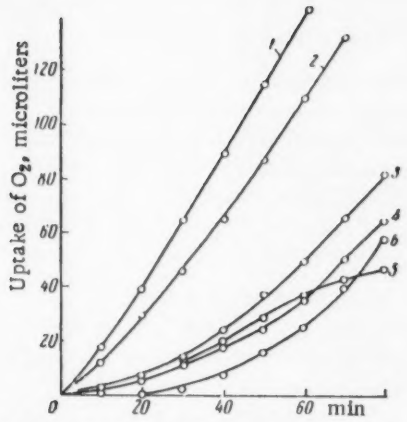


Fig. 2. Oxidation of organic acids of the citric acid cycle by suspensions of BS N-1235. Each sample contained 35 mg dry weight of bacteria and 50 μmoles of substrate; 1) glycerol; 2) fumarate; 3) citrate; 4) α-ketoglutarate; 5) succinate; 6) glutamate. Phosphate buffer – 0.1 M, pH 7.0; added – 0.2 ml 10% KOH at 35°. Uptake of O_2 calculated after deducting endogenous respiration.

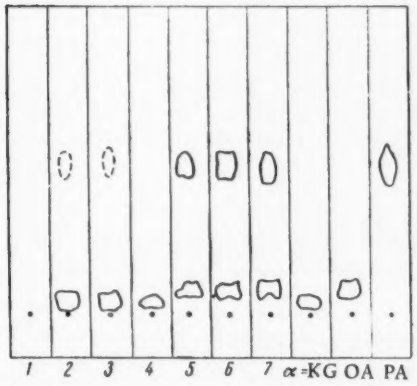


Fig. 3. Chromatographic determination of α-ketoacids formed in the oxidation of organic and amino acids. Solvent: n-butanol saturated with 3% aqueous ammonia. Substrate: 1) endogenous respiration; 2) citrate; 3) KG; 4) L-GL; 5) succinate; 6) fumarate; 7) DL-AS. Last three strips – 2,4-dinotrophenylhydrazones of ketoacids ("indicators").

media with different sources of N and C (Table and Fig. 1). None of the five strains of "wild" BS studied gave normal growth on the basic ammonium medium with glucose or glycerol. In some experiments with this medium, the addition of bicarbonate to neutralize the acids formed in the culture of glucose and glycerol also assured the presence of enough CO_2 for carboxylation of PA [5, 6]; under these conditions BS used this source of C, but the rate of growth was slight. In studying the organic acids of the CAC as sources of carbon nutritives, it was found that citrate or PA maintained the growth of BS N-1235, while KG, succinate, or fumarate did not assure growth. When small amounts of glycerol were added to the medium with NH_4^+ andsuccinate or fumarate there was distinct growth; in media with NH_4^+ + KG + glycerol, growth was satisfactory. L-GL was a good source of C and N, and L-AL was utilized with about the same effectiveness as L-GL. D-GL alone gave weak growth, but in the presence of glycerol, very good growth.

We also studied the growth of BS N-1235 with time on media with different combinations of C and N sources.

As Fig. 1 shows, growth is completely absent on media with only DL-AS and with NH_4^+ in the presence of glucose, glycerol, succinate, fumarate, or KG. The greatest rate of growth in the exponential phase (the same in all cases) and the earliest beginning of growth occur when along with GL, AS, or NH_4^+ ions the medium contains citrate, succinate, or fumarate with supplementary amounts of glycerol added. If L-GL serves as the sole source of C and N, the latent phase is lengthened (from 8 to 13 hours) and the rate of exponential growth, and also the total amount of growth, fall (Fig. 1, curve 8); an analogous growth curve is obtained with media which contain KG, NH_4^+, and glycerol (curve 11). Considerable lengthening of the latent phase and then rapid exponential growth is observed when BS N-1235 is grown on L-AL alone or on citrate with NH_4^+ (Fig. 1, curve 7 and 3).

The above experiments show that BS ("wild" strain) can use NH_4^+ for growth but only under conditions of supplying a suitable source of carbon. The absence or low rate of growth of these bacilli on some sources of C can be explained by the fact that the microbe cells do not form from these compounds with sufficient speed the carbon skeleton which is essential for the synthesis of amino acids.

Oxidation of respiratory substrates. The path of oxidation of substrates of cellular respiration in BS has been studied by several authors [3, 11], but the results do not agree fully. Thus, for example, Sewell and King [11] have assumed the presence in BS of the Thunberg-Knoop dicarboxylic acid cycle, about which other authors have expressed doubt.

In studying the oxidation of respiratory metabolites by a thick suspension of BS N-1235 cells we found, in agreement with the data of Piérard [3] on the Am^- mutant of BS that all the acids which belong to the CAC undergo oxidation (Fig. 2). However, the initial rate of oxidation of some metabolites (especially succinic, citric, ketoglutaric and glutamic acids) is low and the curve of uptake

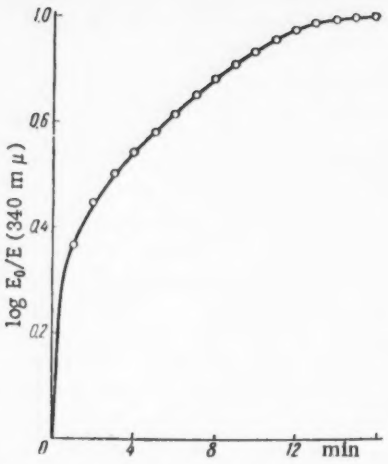


Fig. 4. Activity of aconitase and isocitrate dehydrogenase in cell-free extracts of BS N-1235. Reaction mixture: enzyme extract (6.5 mg protein); 10 μmoles citrate; 0.6 μmoles TPN; 1.0 ml phosphate buffer (0.1 M, pH 7.5); total volume 3 ml.

of O_2 usually shows a constant rise of the autocatalytic type. Oxidation of GL begins after a latent period (about 20 min); the rest of the curves of oxidation of GL and KG are the same.

α-Ketoacids formed in oxidation of CAC metabolites by washed BS cells were identified chromatographically (Fig. 3). When the respiratory substrate was citrate or KG, KG was chiefly observed in the incubation mixture; sometimes we could detect small amounts of PA. In the oxidation of succinate, fumarate, or AS the cell suspension showed oxaloacetic acid and PA; oxidation of GL was accompanied by formation of KG.

Spectrophotometric investigation of the reduction of TPN in the presence of citrate permitted us to establish the presence of a highly active aconitase and isocitrate dehydrogenase in the cell-free extract of BS (Fig. 4).

In connection with the fact that on a basic ammonium medium strain N-1235 grows with supply of carbon in the form of citrate (but not in the form of KG), we tried to detect the presence of a second, parallel path of breakdown of citrate. In orienting experiments we did not obtain results which confirmed the anaerobic splitting of citric acid into C_4 and C_2 fragments, but the presence of such a mechanism in BS cannot be positively excluded.

Products of alanine metabolism in growing BS cultures. The presence of AD-ase permits a normal BS strain to carry out both reductive amination of PA and the transformation of AL into NH_4^+ and PA which undergoes further oxidation through the CAC. As already mentioned, Wiame and others [2, 3] could not establish the role of AD-ase and AL in the process of nitrogen assimilation in BS, in view of the inability of the Am^- mutant of this form to grow in a medium with AL or with NH_4^+ and glycerol.

Chromatographic analysis of the culture liquid at the time of exponential growth of strain N-1235 on a medium with AL as the sole source of C and N showed accumulation of PA, KG and GL, and slight amounts of AS in the medium (Fig. 5). It is quite clear that PA, which arises in the oxidative deamination of AL, is transformed through the CAC into KG and oxaloacetic acid; reamination of these ketoacids with the remaining AL leads to formation of GL, AS and new quantities of PA.

The results of studying the conditions of growth and the oxidative metabolism thus confirmed the presence of the whole CA cycle in the normal strain. However, it was shown that the rate of some intermediate steps in the path of reciprocal transformations of KG and PA was relatively low and could limit or even prevent the multiplication of BS in a definite synthetic medium.

Growth and oxidative metabolism of mutant BS strains. When our study was approaching its conclusion, we obtained some mutant strains of BS, kindly supplied by Prof. Wiame, including the Am^+ mutants IRC-1 and IRC-7, and some Am^- mutants (IRC-6 and others).

In a few of the experiments which we carried out on the growth and characteristics of metabolism in the strain IRC-6, we obtained results which confirmed and supplemented the findings of Wiame and his co-workers. According to the data of Wiame, this strain, like the other Am^- mutants, could not grow in the absence of GL, even in a medium with AL and citrate. When they grew in a medium with GL as the sole source of C and N, these mutants oxidized GL with accumulation of NH_4^+, although they did not have GD-ase [3]. The required use by the Am^- strain of GL (or the corresponding proline, ornithine, or arginine) led Wiame to the conclusion that the assimilation of NH_4^+ through AL in these organisms did not occur. He assumed that the basic function of AD-ase was to assure the indirect deamination of GL with participation of GL-AL-transaminase and AD-ase. This hypothesis is clearly groundless; it is directly contradicted by the reversibility of the reaction catalyzed by these enzymes. If the hypothesis of Wiame of the path of oxidation of GL were correct, then the Am^- mutant of type IR-6 should multiply in a medium which contained AL as the source of C and N instead of GL; it should also grow on NH_4^+ with PA.

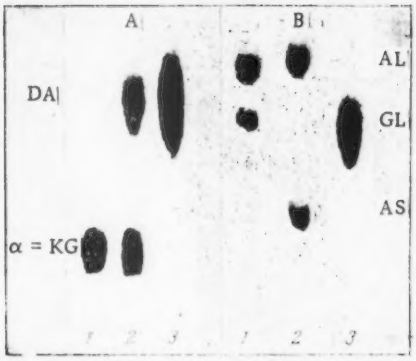


Fig. 5. Chromatographic determination of the products of metabolism of L-alanine in the culture liquid from Bac. subtilis N-1235. A: 1) KG 2,4-dinitrophenylhydrazone; 2) culture liquid; 3) PA. Separation carried out in butanol with ammonia; for developing the ketoacid spots the chromatogram was sprayed with 2% NaOH solution. B: 1) culture liquid; 2) AL (upper spot), AS (lower spot); 3) GL. Separation carried out in a mixture of butanol-formic acid-water (75:15:10); to detect the amino acids the chromatogram was developed with 0.5% ninhydrin in acetone.

Actually we found that AL or PA with NH_4^+ did not maintain growth of BS by IRC-6 even when KG was added to the medium. Also, cells of the mutant IRC-6 (thick seeding) with 20 hour aerobic incubation in a medium with only AL produced only PA and KG, but in distinction from strain N-1235 (see above) did not form GL. The conclusions from these findings are given below.

DISCUSSION

The data given in this and the preceding works [1] give all the reasons for assuming that in BS and probably in other forms of the genus Bacillus which do not have GD-ase, the assimilation of ammonia is attained by alterations in the mechanism of transamination. This mechanism differs from the corresponding processes in other organisms [9, 10] in that in it the first step in the assimilation of NH_4^+ is the amination of PA catalyzed by AD-ase which is followed by the transfer of the NH_2 group of AL to KG and further distribution of the nitrogen by transamination reaction and rebuilding of side chains of amino acids.

Study of the conditions of growth of BS N-1235 showed that for the development of this microorganism on an ammonia based medium the nature of the source of carbon was decisive. The effect of the source of C on the utilization of NH_4^+ by the microorganism has been noted frequently, for example for BS [5, 6] and for bacteria of acetic acid fermentation [12, 13].

The results of the study of growth conditions and metabolic processes of BS N-1235 can be summarized and explained as follows:

1. These bacteria grow on a medium with NH_4^+ and PA, but do not grow when the source of the carbon is glucose or glycerol. This is explained largely by the fact that glycerol and glucose in the culture form acid decomposition products which remove from the medium the CO_2 needed for the carboxylation reaction; when bicarbonate is added to such a medium, moderate growth is observed (see [4-6]).

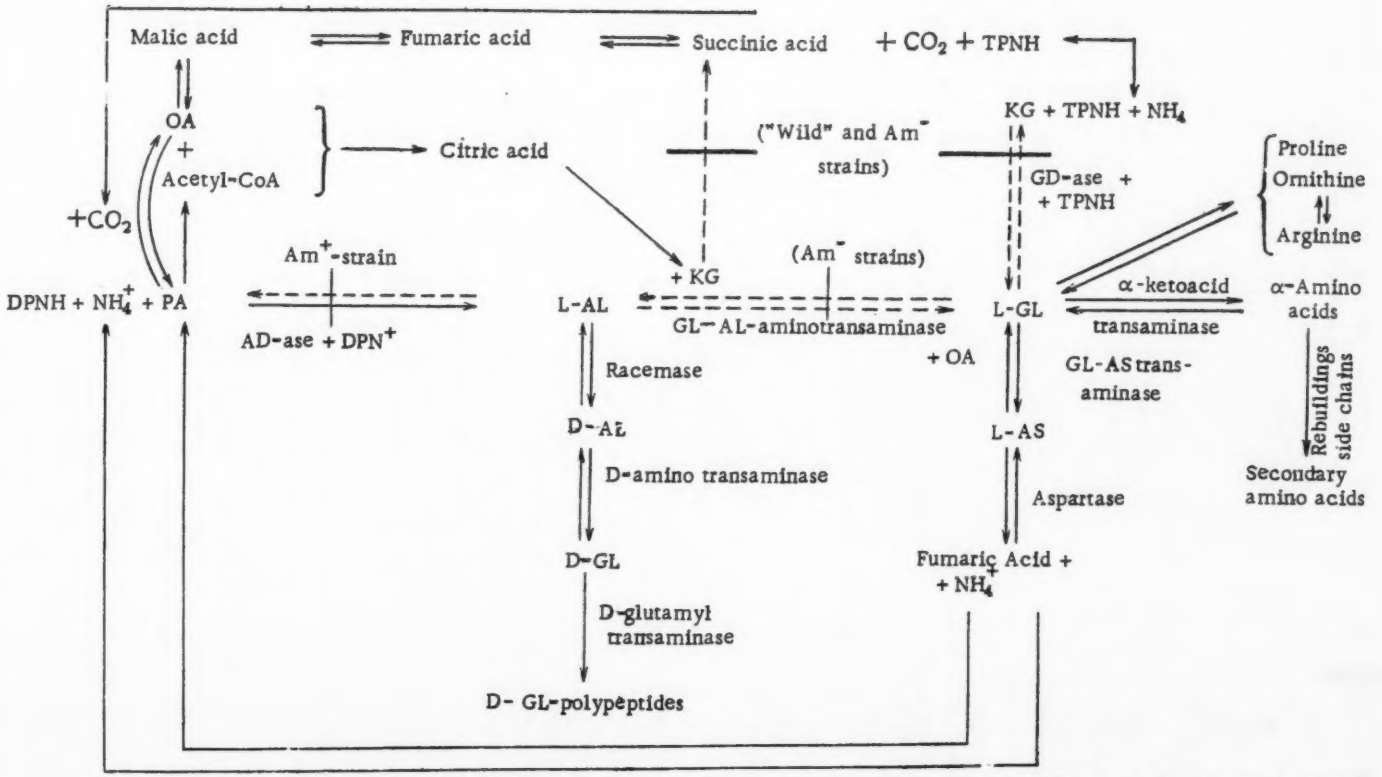
2. Strain BS N-1235 does not multiply in a medium with only ASC though it contains aspartase) or on an ammonia medium with succinate, fumarate, or KG as the source of C; when a certain amount of glycerol is added, rapid and abundant growth occurs on these media (the effect of glycerol is most clearly shown in media with KG).

The initial rate of oxidation by the cells of BS N-1235 of many di- and tricarboxylic acids related to the CAC is low enough (Fig. 2) as Piérard also found in experiments with an Am^- mutant of BS [3].

In a suspension of BS N-1235 in the oxidation of citrate, GL, and KG there is no perceptible amount of PA. All these results show that the unfavorable effect of the dicarboxylic acids of the respiratory cycle as the sole source of carbon for BS can be explained by the insufficient rate of one or another step in their transformation to PA, especially the low activity of the system KG-oxidase.

3. Differing from KG, citrate maintains the growth of these microbes on an ammonia medium. There is then a long latent phase which can be shortened by adding glycerol. In orienting experiments we did not obtain results to show the splitting of citrate in extracts of BS N-1235 into C_4 and C_2 fragments, but the presence of such a transformation is not excluded.

The sharp difference in utilization of citrate by these strains on the one hand, and of KG on the other, as the sole source of carbon, may be connected with the fact that for each mole of substrate from KG oxidized by the cells there is formed less CO_2 for the carboxylation reaction) and less hydrogen (for reductive amination) than from citrate. The problem raised by this point requires further investigation.



Scheme of Basic Processes of Amino Acid Synthesis and Deamination in Bac. subtilis. Note: The dotted arrows indicate transformations whose rate is small and in some strains is insufficient. The arrows crossed by a line mean transformations which are absent or very weak in the strains shown in the scheme.

4. Both Al and GL can be used by strain N-1235 as the sole source of N and C, but the latent phase is then prolonged and the rate of growth is slight. The optimum conditions for growth are assured by adding fumarate (or other dicarboxylic metabolite) to the AL, or, correspondingly, by adding glycerol (or PA) to the medium with GL. These observations confirm the conclusion that optimum growth of BS (normal strains) on an ammonia medium is possible only when there is present in the medium as a source of carbon nutrient an assured abundant presence (or formation) of PA or KG which are successive acceptors of nitrogen in the synthesis of amino acids by the bacillus. Therefore, exactly the best conditions for growth of BS are given by the simultaneous presence in the medium of PA (or its precursors) and dicarboxylic metabolites of the respiratory cycle.

5. The hypothesis of Wiame [3] that in the mutant IRC-6 (Am^-, GD-ase^-, AD-ase^+) the oxidation of GL occurs by transdeamination with the participation of GL-AL-transaminase and AD-ase is evidently incorrect. This assumption obviously is in contradiction to the reversibility of both these enzymes and to the inability of BS IRC-6 to grow on AL or on NH_4^+ and PA, even in the presence of KG. In our opinion, the most probable path of GL oxidation in this mutant and in other forms of the genus Bacillus which do not have GD-ase is the indirect deamination of GL under the influence of GL-AL-transaminase and aspartase. The participation of aspartase in the biosynthesis of amino acids has not been established for any organism and we share the view of Wiame [4] according to which the function of aspartase in metabolism in bacteria is connected more with nitrogenous catabolism than with assimilation of ammonia.

6. Strain IRC-6, in distinction to BS N-1235, does not form GL among the products of AL metabolism. This fact and all the related data on the conditions of growth of this strain and especially its metabolism [1, 3, 4, 7] gives us reason to suppose that the inability of BS IRC-6 and perhaps some Am^- mutants closely related to it to assimilate NH_4^+ nitrogen or alanine depends on the great lack (if not full absence) of the transamination reaction, direct or stepwise, by which the amino group of AL is transferred to KG. In sum, there is no reason to think that the decisive defect in all the Am^- mutants of BS is the same.

We give the chief paths of synthesis of amino acids and deaminations in Bac. subtilis which have been deduced from the main results of this and the previous [1] work, and also the data of the school of Wiame in the scheme shown on page 886.

SUMMARY

In order to explain the mechanism of assimilation of ammonia in Bac. subtilis we have studied the conditions of growth (use and source of nitrogen and carbon) and the characteristics of metabolism of the respiratory substrates in the normal strain BS N-1235.

For optimum growth of this strain in a basic medium with ammonium salts as the source of nitrogen there must be simultaneous presence in the medium of pyruvic acid or alanine (or their precursors such as glucose, glycerol) and ketoglutaric acid (or other di- and tricarboxylic acids of the citric acid cycle).

The reason for the absence or small intensity of growth of BS N-1235 on an ammonia medium with various sources of carbon is the insufficient rate of some reactions in the path of the reciprocal transformations of pyruvate and ketoglutarate in the respiratory cycle.

The data of this and the preceding [1] work show that the basic path of assimilation of ammonia nitrogen in BS (and in other bacilli which lack GD-ase) is the alteration in the process of transamination. The first stage in this process is the amination of pyruvate by AD-ase and the second step is the transfer of the amino group from alanine to ketoglutarate, after which follows the formation of other amino acids by ordinary pathways (transamination, rebuilding of side chain amino acids).

In this work we have discussed details of the synthesis and deamination of amino acids in normal and Am^- strains of BS. The idea has been presented that the basic metabolic defect in the mutant of Wiame and Piérard which cannot assimilate ammonia is the destruction of the formation of L-glutamic acid from L-alanine and α-ketoglutaric acid.

LITERATURE CITED

[1] M. M. Hung, S. C. Shen and A. E. Braunshtein, Biokhimiya 24, 929 (1959); Biochim et biophys. acta 36, 288 (1959).

[2] J. M. Wiame, J. Collette and S. Bourgeois, Arch. internat. physiol. biochim. 63, 271 (1956).

[3] J. M. Wiame and A. Piérard, Nature 176, 1073 (1955); A. Piérard, These de Doctorat, Bruxelles (1958).

[4] J. M. Wiame, Advances Enzymol. 18, 241 (1957).

[5] J. M. Wiame and S. Bourgeois, Biochim. et biophys. acta 18, 269 (1955).

[6] J. M. Wiame, C. Rozenblum and S. Bourgeois, Biochim. et biophys. acta 20, 43 (1956).

[7] J. M. Wiame and R. Storck, Biochim. et biophys. acta 10, 268 (1953).

[8] D. Cavallini, N. Frontali-Toschi, and G. Toschi, Nature 163, 568 (1953).

[9] A. E. Braunshtein, Chief Paths of Assimilation and Deamination of Nitrogen in Animals (XII Bakh Lecture) Izd. AN SSSR,(Moscow, 1957); Advances Enzymol. 19, 335 (1957).

[10] A. E. Braunshtein and R. M. Azarkh, Biokhimiya 22, 430 (1957); Arch. Biochem. and Biophys. 69, 634 (1957).

[11] C. E. Sewell and K. H. King, Biochem. J. 58, XLIII (1954).

[12] A. N. Hall, K. S. Tewari, G. A. Thomas and T. K. Walker, Arch. Biochem. and Biophys. 46, 485 (1953).

[13] M. R. R. Rao and J. L. Stokes, J. Bacteriol. 65, 405 (1953).

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THE EFFECT OF TOTAL X-RAY IRRADIATION ON THE CONTENT OF GLYCOGEN IN THE RAT LIVER

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One of the most important results of the action of penetrating radiation on the animal organism is the destruction of metabolism in the widest sense of this word, and in particular, the destruction of carbohydrate metabolism. There is great interest in studies attempting to elucidate the possibility of suppression of synthetic processes in the irradiated organism and the predominance of processes of breakdown [1]. From this point of view, the study of the processes of glycogen formation in liver tissue from the dynamic aspect, in all stages of development of radiation sickness, is of definite interest.

METHODS

The state of the glycogen forming function of the liver was estimated by growth in glycogen content in liver tissues of starving rats after loading with glucose. The experiments were carried out on white rats of both sexes with a weight of 190-200 g. After 24 hour starvation the rats were injected subcutaneously with a solution of glucose calculated at 1 g per 100 g body weight. After four hours the animals were decapitated. The liver tissues were removed rapidly, frozen with liquid air and by the method of van der Klei the glycogen content was determined [2]. The experimental rats were starved for 24 hours, then they were submitted to total x-ray irradiation in an RUM-3 apparatus with an absolutely lethal dose (1000 r). Conditions of irradiation: voltage 190 kv, current strength 20 ma, skin focus distance 30 cm, filter 0.5 mm copper and 1.0 mm aluminum, half weakening layer 0.75 mm copper, intensity of dose in air 72.2 r/min.

The state of the glycogen forming function of the liver was studied before irradiation, immediately after it, and at 2, 4, 24, 48 and 96 hours after irradiation had stopped. The control studies for each group of experimental rats were carried out on unirradiated animals. The results, treated statistically, are shown in the table. The error of the mean was calculated by the formula:

$$M_1 = \pm \sqrt{\frac{\frac{\Sigma a^2}{n} - \left(\frac{\Sigma a}{n}\right)^2}{n}},$$

where M_1 is the error of the mean, a, the variation from the arithmetical mean, and n the number of experiments.

RESULTS AND DISCUSSION

In all controls (unirradiated) without exception, the rats assimilated glucose and deposited it normally as glycogen: four hours after the glucose injection the content of glycogen in the liver tissues of unirradiated animals rose on the average from 401 to 1977 mg %.

After subcutaneous injection of glucose in irradiated rats there was a clear inhibition in their liver of the

Glycogen Content in Liver of Irradiated and Control Rats after Glucose Loading

Experimental conditions	Number of animals	Glycogen content, mg% ($M \pm m_1$)	Change compared to controls, %
Control			
24 hour starvation	29	401±1,43	—
The same + subcutaneous glucose	40	1977±113,8	—
Irradiated (1000 r + glucose subcutaneously)			
Directly after irradiation	22	1772±31,8	—10
The same	12	737±1,9	—62
After irradiation, hours			
2	16	551± 9,04	—72
2	3	2565± 29,5	+30
4	11	2115±137,6	+ 7
4	7	462± 46,8	—77
24	20	2527± 86,3	+28
48	11	1952± 80,8	— 1
96	17	662± 40,5	—67

processes of glycogen formation. This effect was even observed directly after irradiation in 35% of the animals and in almost all of the animals (84%) two hours after the irradiation. The glycogen level of the liver in these rats after thay had been injected subcutaneously with glucose was about 70% below that of the unirradiated rats, on the average, 551-737 mg%. When the period after irradiation is longer, the low content of glycogen in the rat liver after glucose injection is considerably rarer. At 24 hours after irradiation the content of glycogen in rat livers with subcutaneous injection of glucose was even somewhat higher, and at 48 hours was the same as in the control (unirradiated) animals. Up to this time other investigators also have found a high glycogen content in the liver of animals irradiated with lethal doses [3-11]. Also, 96 hours after irradiation, subcutaneous injection of glucose does not produce any notable increase in glycogen storage in the liver: its content was about the same as in rats which had not received glucose (on the average 401 and up to 662 mg% glycogen after injecting glucose).

From a consideration of such results it is evident that in the irradiated animals there is actually a destruction of the glycogen forming function of the liver: in spite of the injection of a sufficient amount of glucose, the content of glycogen in the liver tissues remains low. This can be clearly seen in short periods after the action of a lethal dose of radiation (immediately or after 2-4 hours) and also in longer periods (after 90 hours). The observations at definite stages in the development of radiation sickness (24-48 hours after irradiation) of normal or somewhat increased deposition of glycogen in the livers of irradiated animals require an explanation.

Various mechanisms can produce this effect. Thus, the accumulation of glycogen in the irradiated organism may occur because of inhibition of glycogen splitting and on account of its synthesis, preferably from amino acids and products of fat metabolism.

The idea of the great slowing of glucose splitting in the irradiated organism finds confirmation in the experiments of Hevesy and Forsberg [12] and also of Morehouse et al [7]. Minaev [13] has also shown the possibility of destruction of mechanisms concerned with the oxidative transformations of glucose.

The point of view of these investigators is also correct which considers the increase of glycogen in animal livers in definite phases of the development of radiation sickness as the result of strengthening glyconeogenesis. Danilova [14] after injecting irradiated rats with cortisone, which stimulates the processes of glyconeogenesis, found a great deposition of glycogen in rat livers at the time when the level of glycogen in the control irradiated animals fell sharply. Obviously, here glycogen was synthesized from compounds formed when the breakdown of fats and proteins was increased [9, 15]. An increase in protein catabolism was considered to be one of the reasons

for this effect by Lourau-Pitres [16]. Hence, when, after irradiation, there is an increased content of carbohydrate in the liver, it is still not necessary to speak of normal occurrence of glycogen formation.

Our results also disprove the idea of some investigators [17, 18] who have tried to explain the slight deposition of glycogen in the liver at definite stages in radiation sickness chiefly by insufficient entry of carbohydrates into the blood stream because of damage to their absorption in the gastro-intestinal tract. Actually, a number of authors [19-24] have showed damage to the functions of the gastro-intestinal tract, and especially a decrease in absorption of carbohydrates in the small intestine after irradiation, and this fact cannot be disregarded in studying the processes of glycogen formation. However, since the plan of our experiments excluded the possibility of lowering the glycogen level in the liver because of insufficient entry to it of carbohydrates from the blood stream (introduction of glucose, not by mouth, but subcutaneously to avoid the gastro-intestinal tract), we are justified in drawing a conclusion about the clearly demonstrated inhibition of glycogen formation in animals. This damage can occur even immediately after irradiating the animals with a lethal dose.

SUMMARY

In control rats starved for 24 hours, the subcutaneous injection of glucose (1 g per 100 g body weight) restores the initial glycogen content in the liver.

In starved rats totally irradiated with a dose of 1000 r there is an inhibition of assimilation of glucose. Immediately after irradiation and also after 2, 4 and 96 hours the glycogen content in the liver in most rats after glucose injection averaged 70% lower than it did in the control animals.

LITERATURE CITED

[1] S. E. Manoilov, Collection: Problems of Radiobiology, 2 (1957).

[2] Van der Klei, Biochim. et biophys. acta 7, 481 (1951).

[3] N. North and L. F. Nims, Federat. Proc. 8, 119 (1949).

[4] R. W. McKee, Federat. Proc. 11, 256 (1952).

[5] J. R. Denson, E. J. Gray, L. D. Gray, F. J. Herbert, J. T. Few and H. Jensen, Proc. Soc. Exptl. Biol. and Med. 82, 707 (1953).

[6] M. Lourau, Compt. rend. Acad. Sci. 236, 422 (1953).

[7] M. G. Morehouse and R. L. Searcy, Science 122, 3160, 158 (1955).

[8] R. E. Kay and C. Entenman, Proc. Soc. Exptl. Biol. and Med. 91, 1 (1956).

[9] R. W. McKee and M. Brin, Arch. Biochem. and Biophys. 61, 390 (1956).

[10] B. M. Graevskaya and R. Ya. Keilina, Vestn. Rentgenol. i Radiol. 4, 21 (1955).

[11] D. A. Golubentsev, Papers of Conference: Early Mechanisms of Radiation Injury p. 27, Khar'kov (1958).

[12] G. Hevesy and A. Forsberg, Nature 168, 4277, 692 (1951).

[13] P. F. Minaev, Zhur. Obshch. Biol. 15, 401 (1954).

[14] E. L. Danilova, in: Radiation Sickness and Related Damage, (Leningrad, 1958) p. 121.

[15] P. Fischer, Arch. internat. physiol. 62, 134 (1954).

[16] M. Lourau-Pitres, Arkiv for kemi 7, 211 (1954).

[17] L. F. Nims and E. Sutton, Amer. J. Physiol. 177, 51 (1954).

[18] I. I. Ivanov, V. S. Balabukha, E. F. Romantsev and T. A. Fedorova, Metabolism in Radiation Sickness, Moscow, 1956.

[19] K. W. Buchwald, Exptl. Med. 53, 827 (1931).

[20] J. O. Ely and M. H. Ross, Williams Wilkins Comp. (Baltimore, 1947) p. 142.

[21] M. Lourau and O. Lartique, Compt. rend. Acad. Sci. 232, 1697 (1951).

[22] H. M. Dickson, Amer. J. Physiol. 182, 47 (1955).

[23] L. E. Detrick, H. C. Upham, D. H. V. Debley and T.J. Haley, Radiation Res. 2, 483 (1955).

[24] S. R. Perepelkin, Papers at the 8th All-Union Congress of Physiol. Biochem. Pharmacol. p. 473, (Moscow, 1955).

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CHANGES IN SOME PHYSICOCHEMICAL PROPERTIES OF GLIADIN UNDER THE INFLUENCE OF HEAT

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The drying of grains (either for seed or for food storage) their hydrothermal treatment is carried out by heating the grain under various conditions of moisture. A study of the action of heat on the grain proteins was undertaken by Kretovich, Lenarski and others [1-5]. However, these investigations were carried out on the proteins found in the grain, that is, on proteins which are in complexes with other substances.

We therefore set up the problem of studying the changes in physicochemical properties of the individual proteins under the influence of heat.

EXPERIMENTAL

Gliadin was used for the study; it was isolated by the method described previously [6] from winter wheat of the variety "Moscow 2453."*

The following characteristics of gliadin were determined: content of total and amino nitrogen; solubility and specific rotation, $[\alpha]_D$ of gliadin in 65% ethanol. Total nitrogen was determined by the method of Kjeldahl in the micromodification; amino nitrogen by the method of Tsuverkalov [7]; specific rotation and solubility by the usual methods.

TABLE 1

Total Nitrogen Content of Gliadin (%) Depending on Moisture, Temperature and Length of Exposure

Moisture, %	Exposure, min	40°	50°	60°	70°
14,2	15	16,94	16,89	16,95	16,96
	30	16,88	16,88	16,73	17,00
	60	16,90	16,96	16,95	16,91
18,1	15	16,90	16,91	16,89	16,98
	30	16,99	16,84	16,82	17,08
	60	16,99	16,73	16,98	16,96
20,4	15	16,80	16,90	16,81	16,90
	30	16,91	16,99	16,87	16,97
	60	17,06	17,01	16,97	17,04

RESULTS

A study of the initial gliadin showed that the content of total nitrogen was 17.01%, amino nitrogen 1.94%, solubility in ethanol 97.86%, $[\alpha]_D$-83.3°.

*The 1957 harvest of wheat was obtained from the K. A. Timiryazev Agricultural Academy.

TABLE 2

Amino Nitrogen Content (%) in Gliadin Depending on Moisture, Temperature and Length of Exposure

Moisture, %	Exposure, min	40°	50°	60°	70°
14,2	15	2,75	2,70	2,65	2,25
	30	2,89	2,83	2,70	2,15
	60	2,96	2,76	2,40	1,78
18,1	15	2,86	2,81	2,50	2,10
	30	2,96	2,80	2,30	1,76
	60	2,93	2,75	2,24	1,58
20,4	15	1,75	1,70	1,70	1,63
	30	1,71	1,70	1,68	1,58
	60	1,70	1,70	1,70	1,57

TABLE 3

Solubility of Gliadin (%) Depending on Moisture, Temperature and Length of Exposure

Moisture, %	Exposure, min	40°	50°	60°	70°
14,2	15	93,51	89,20	87,71	84,29
	30	90,61	87,42	84,22	81,91
	60	89,39	81,67	77,29	75,19
18,1	15	88,99	77,10	67,34	59,15
	30	80,69	70,93	62,24	55,33
	60	77,85	66,50	56,29	48,78
20,4	15	74,32	67,54	56,87	36,84
	30	69,69	61,38	50,45	28,43
	60	58,11	51,30	42,25	27,44

To study the action of heat on the protein with varying moisture content, we moistened the isolated gliadin. As a result we obtained samples of protein with differing moisture: 14.2, 18.1 and 20.4%. The moisture of the protein during heating remained constant. Heating was carried out at 40, 50, 60 and 70° for 15, 30 and 60 min. Thus, we obtained 36 types of gliadin. The average data for each test from four or five repetitions are given in Tables 1-4.

Table 1 shows that the content of total nitrogen in gliadin under the conditions which we studied remains constant. At the same time, the change in moisture, temperature and length of exposure has a considerable effect on the amino nitrogen content of gliadin (Table 2).

The data of Table 2 show that keeping the gliadin with moisture of 14.2 and 18.1% at 40 or 50° causes an increase in amino nitrogen, while the action of higher temperature (60, 70°) has a reverse effect.

The action of heat on gliadin with a moisture of 20.4% causes some decrease in amino nitrogen at the beginning (after 15 min at 40°); with further increase in temperature and length of exposure, the amino nitrogen content is practically unchanged. The action of heat on gliadin leads not only to a change in the amount of amino nitrogen in it, but to a change in its physical properties: solubility and specific rotation (Tables 3 and 4).

The higher the moisture of the preparation and the temperature at which the exposure was carried out, the greater the lowering of gliadin solubility. A similar relation is found between the temperature and optical density of the preparation. Initial action of heat causes a slight increase in specific rotation of the gliadin. However, at 50 and 60° the optical activity rises sharply. Further increase in temperature causes lowering of specific rotation of gliadin. Depending on the moisture of the preparation the fall in specific rotation is greater or less.

TABLE 4

Specific Rotation of Gliadin (in degrees) Depending on Moisture, Temperature and Length of Exposure

Moisture, %	Exposure, min	40°	50°	60°	70°
14,2	15	— 92,5	—102,0	—103,8	—101,2
	30	— 97,0	—110,8	—115,6	—111,4
	60	—105,3	—118,9	—120,1	—112,6
18,1	15	— 90,1	—102,6	—104,3	— 97,5
	30	— 95,5	—106,0	—110,1	— 97,0
	60	— 92,5	—104,2	—107,0	— 95,6
20,4	15	— 92,1	—106,5	—112,0	— 99,1
	30	— 94,7	—105,0	—108,1	— 93,4
	60	— 88,7	—100,0	—104,2	— 92,5

Thus, the change in specific rotation of gliadin, like the fall in its solubility, depends chiefly on the moisture of the protein and the action of temperature.

DISCUSSION OF RESULTS

On the basis of the results which we have presented, we can conclude that the properties of gliadin (amino nitrogen, solubility, specific rotation) change with the temperature at which the preparation is kept and with the moisture of the protein and the length of action of heat. Moisture and temperature show a stronger action on the properties of gliadin; the relation of change of properties with time of heat action is much smaller. The change in content of amino nitrogen is especially clear and at once shows the relation of the gliadin properties to the three factors mentioned.

The rise in amino nitrogen in the initial stages of heat action (40, 50°) suggests that the gliadin molecule, undergoing denaturative changes [8-10], splits under these conditions and forms new amino groups. With further action of heat there are changes in the protein molecule which are probably deeper. As a result of these changes there are probably formed some new bonds, and the amino nitrogen content falls, becoming stabilized at a definite value. Popadich [11] testing the action of heat on gluten, also found a fall in amino nitrogen.

Our suggestion of the behavior of the gliadin protein molecule under the influence of heat is confirmed by the results on change in the physical properties of gliadin. The solubility of gliadin is clearly related to all three factors: moisture, temperature and length of exposure, with moisture and temperature playing the chief roles.

The considerable fall in solubility of gliadin after heat denaturation in our experiments is evidently connected with the decreased number of polar groups (as, for example, amino groups) in the deep structural changes of the protein molecule.

Similar conclusions are reached on the basis of the data on change in specific rotation of gliadin under the action of heat. Since the change in specific rotation shows changes in the structure of the protein molecule [12, 13] these data confirm our suggestion of the behavior of gliadin under the influence of heat. Evidently, the initial action of heat causes splitting of the gliadin protein molecule which leads to increased specific rotation. More intense action of heat apparently causes formation of new bonds and thus racemization and fall in specific rotation.

The absence of change in total nitrogen content of gliadin under our conditions probably means that no side products are formed in this process.

SUMMARY

In gliadin preparations with different moisture content (14.2, 18.1, 20.4%) the content of total nitrogen does not change with heat (heating at 40, 50, 60 and 70° for 15, 30 and 60 min).

Changes in the physicochemical properties of gliadin under the influence of heat depend on the moisture content of the preparation and on temperature and length of its action; moisture and temperature play the chief roles. The content of amino nitrogen under the influence of heat in gliadin with moisture content of 14.2 and 18.1% at first rises and then falls, while with moisture content of 20.4% there is a fall at all temperatures. The solubility of gliadin falls more sharply at higher temperatures, moisture and exposure.

The specific rotation of gliadin with moisture content 14.2, 18.1 and 20.4% rises under the influence of heat at 40, 50 and 60° and falls at 70°.

LITERATURE CITED

[1] V. L. Kretovich, A. A. Bundel', T. L. Smirnova et al., Biochemistry of Cereals, No. 2, 45 (1954).

[2] V. L. Kretovich and T. G. Florenskaya, Biochemistry of Cereals, No. 4, 56 (1958).

[3] I. L. Lenarskii, Biochemistry of Cereals No. 1, 114 (1951).

[4] L. L. Lenarskii, Trudy Odesskogo Tekhnologicheskogo In-ta No. 5, 36 (1955).

[5] J. W. Pence, A. Mohammad and D. K. Mecham, Cereal Chem. 30, 115 (1953).

[6] E. L. Medvedeva, Reports of Higher Educational Institutes, Ministry of Higher Education, Food Technology, No. 4, 163 (1958).

[7] D. Tsuverkalov, Biokhimiya 6, 243 (1941).

[8] L. N. Bulankin, in: Proteins, Their Specific Properties, p. 186, Izd. AN UkrSSR (Kiev, 1955).

[9] A. G. Pasynskii, in: Proteins, Their Specific Properties, p. 144, Izd. AN UkrSSR (Kiev 1955).

[10] F. Putnam, in: Proteins [Russian translation] 2 (IL, Moscow, 1956) p. 602.

[11] I. A. Popadich, Khlebopekarnaya i Konditerskaya Promyshlennost' No. 1, 21 (1957).

[12] P. Doty and E. Jedushek, in: Proteins (IL, Moscow, 1956) 2, p. 363 [Russian translation].

[13] J. A. Schellman, C. Schellman and Green, Arch. Biochem. and Biophys. 65, 58 (1856).

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THE EFFECT OF PLUTONIUM ON THE CONTENT AND RENEWAL OF NUCLEIC ACIDS IN SOME RABBIT TISSUES

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A considerable number of reports have been published in the literature on the effect of a single application of x-rays and γ-rays on the amount and synthesis of nucleic acids in the tissues [1-7], but there has been almost no investigation of the action of deposited radioactive substances and the effects of chronic irradiation on these very important biological compounds. We have considered it important to study the effect of one of these radioactive elements, Pu^{239} on the content and metabolism of nucleic acids in some rabbit tissues.

METHODS

Rabbits of the "Chinchilla" type, of both sexes aged 6-7 months, were injected intravenously with a solution of plutonium nitrate in the amount of 7 μcurie/kg (pH 2.0). After 15 days, 1, 3, and 6 months after injecting the radioactive substance, the animals were killed and the tissues studied (liver, spleen, bone marrow) to determine the amount of nucleic acids by the method of Schmidt-Thannhauser in the modification of Davidson [8]. For the determination of the rate of renewal of the nucleic acids (by phosphorus) the rabbits were injected subcutaneously four hours before killing with a neutral solution of $Na_2HP^{32}O_4$ in the amount of 60 μcurie/kg weight of animal. The rabbits were killed by air embolism, the important tissues were quickly removed, and all further treatment was carried out in the cold. The activity of bone marrow depolymerase was studied by the method of Schneider and Hogeboom [9] for 6 months after the injection of Pu.

In this study we used 16 experimental and 12 control animals of the same age. On the basis of the experimental data as to content of plutonium in the liver, spleen, and bone marrow we calculated the ionizing dose [10]. All the data were submitted to statistical treatment and are given in the form of averages and errors of the arithmetical average. The differences between the data for experimental and control animals were taken as significant at percent error (p) ≤ 0.05 [11].

RESULTS

Injection of the dose of plutonium (7 μcurie/kg) caused some fall in weight of the animals, a decrease in total leucocyte content in the peripheral blood beginning even at 15-30 days and death of most of the animals 6-8 months after injection of the radioactive substance. In distinction from rats [12], Pu in rabbits is stored in much greater amount in the liver (Table 1), and, which is especially important, is retained for a long time in this organ (after 6 months, 41.2% of the injected dose). In this respect the ionization dose entering the liver is very high; a considerable dose of ionization is also found in the tissues of the spleen (Table 1).

As the data of Table 1 show, after 90 and 180 days the weight of the liver of the experimental animals was greatly decreased, falling to 51.9 and 63% of that of the controls of equal age. In this period there was also a definite decrease in ratio of weight of the liver to weight of the rabbit. A definite change in amount of total nitrogen and phosphorus in the liver or bone marrow was not observed.

In Table 2 we give information on the amount of nucleic acids, based on phosphorus, per 1 g of wet tissue, and the specific activity of these compounds. Analysis of the results shown in this table indicates that the RNA content in the liver of the experimental animals was somewhat increased (by 22%) after two weeks and fell three

TABLE 1

Weight of Organs, Amount of Pu, Ionization Dose, and Content of Total N and P in Rabbit Tissues after Intravenous Injection of $7 \cdot 10^{-3}$ mcurie/g of Plutonium Nitrate

Group of animals	Period of study, days	Weight of organs, g	Wt. of organs in % of body weight	Amt. of Pu in organs in % of injected dose	Ionization dose, ber*	Total nitrogen, mg/g	P_{total} mg/g
Liver							
Control	30	102,4±15,8	3,0±0,4	—	—	26,40±0,50	3,31
Expt.	15	98,7±14,2	3,5±0,4	55,4±4,4	4500	25,50±0,37	3,37±0,10
"	30	78,1± 5,9	2,9±0,2	54,5±5,1	9700	23,60±0,42	3,85±0,15
Control	90	86,1± 7,5	2,9±0,3	—	—	25,00±0,88	3,50±0,22
Expt.	90	44,7± 6,4	2,4±0,3	31,4±4,7	33 100	27,00±0,76	4,32±0,25
Control	180	105,2± 9,8	3,4±0,4	—	—	25,80±0,79	3,78±0,03
Expt.	180	66,3± 4,1	2,3±0,2	41,2±3,0	66 400	23,50±0,81	3,17±0,18
Spleen							
Control	30	2,11±0,57	0,062±0,015	—	—	—	—
Expt.	15	1,08±0,21	0,037±0,006	0,25	1950	—	—
"	30	1,30±0,27	0,049±0,008	0,67	5550	—	—
Control	90	1,95±0,51	0,066±0,018	—	—	—	—
Expt.	90	2,18±0,37	0,102±0,014	0,65	18 150	—	—
Control	180	1,60±0,17	0,051±0,008	—	—	—	—
Expt.	180	2,46±0,57	0,083±0,017	0,34	28 950	—	—
Bone Marrow							
Control	30	—	—	—	—	22,35±0,71	2,76±0,37
Expt.	15	—	—	—	139**	—	—
"	30	—	—	—	320	20,20±1,26	2,23±0,14
"	90	—	—	—	1210	20,23±0,64	2,67±0,16
Control	180	—	—	—	—	19,61±0,29	2,31±0,18
Expt.	180	—	—	—	2720	16,52±1,50	2,22±0,35

*ber — biological equivalent of roentgens (for α-particles, 1 per corresponds to 10 ber).

**In calculating the ionization dose for bone marrow we made the assumption that only 1/4 of the α-particles irradiate the bone marrow of the diaphysis of tubular bone, from which also the main amount of tissue was removed. We did not consider that only the part of the bone marrow adjacent to the endosteum underwent irradiation.

months (by 28%) after the Pu injection. After 6 months the amount of RNA in the experimental rabbits did not differ from that of the controls.

The DNA content in the liver changed only after 6 months from the injection of Pu and was 65% of the control. Since a more accurate expression of the content of nucleic acids in the tissues is given by that in cells or organs, we also give the amount of RNA and DNA phosphorus calculated on the weight of the liver (Fig. 1). These data indicate the very sharp fall in RNA content in the whole liver 3 and 6 months after injection of Pu (by 56 and 36% respectively). The fall in amount of DNA phosphorus in this period was also considerable (by 45 and 59%). The ratio of P-RNA/P-DNA increased, compared to the normal during most of the period studied.

There is definite interest in the data which characterize the specific activity of the nucleic acid P of the liver (Table 2). Thus, 3 months after injection of Pu there is a clear increase in specific activity of RNA (2.5 times) which accompanies the simultaneous decrease in its amount. The specific activity of DNA increases sharply during the experiments: a month after injection of the plutonium it had increased 3.6 times, three months after injection, 5.5 times, and after half a year, it was 15 times greater than in the control animals.

Definite changes were found also in the spleen nucleic acids. The P-RNA content fell two weeks after injection of Pu (by 16%) and remained low for about 3 months (by 26%). The amount of P-DNA was decreased for the whole period except at first (2 weeks), on the average by 25-30%. In calculating the nucleic acid content on the weight of the spleen we could not establish clearly changes because of variations in the weight of the

TABLE 2

Content and Specific Activity of Nucleic Acid Phosphorus in Rabbit Tissues at Various Periods after Injection of Plutonium Nitrate

Group of animals	Time after injection of Pu, days	P content in mg/g wet tissue		P-RNA / P-DNA	Specific activity*	
		P-RNA	DNA		RNA	DNA
Liver						
Control	30	0,69±0,028	0,25±0,023	2,83±0,30	10,5±2,02	1,7±0,08
Experiment	15	0,83±0,054	0,29±0,028	3,47±0,35	—	—
"	30	0,71±0,032	0,23±0,028	3,05±0,21	8,2±0,54	6,4±1,20
Control	90	0,74±0,072	0,23±0,025	3,18±0,56	8,6±2,44	1,5±0,23
Experiment	90	0,53±0,055	0,23±0,019	2,38±0,32	21,7±5,55	8,3±1,57
Control	180	0,58±0,015	0,25±0,00	2,84±0,33	7,6±1,25	1,4±0,30
Experiment	180	0,69±0,024	0,15±0,018	4,59±0,49	10,5±1,91	21,1±4,47
Spleen						
Control	30	0,70±0,038	0,94±0,021	0,74±0,014	19,5±1,72	5,6±1,15
Experiment	15	0,50±0,045	1,00±0,031	0,50±0,049	—	—
"	30	0,51±0,052	0,64±0,084	0,87±0,039	31,6±4,90	10,2±1,87
Control	90	0,72±0,053	1,03±0,121	0,71±0,034	23,0±3,40	5,5±0,43
Experiment	90	0,53±0,048	0,73±0,032	0,70±0,050	33,5±4,68	8,9±0,43
Control	180	0,64±0,014	0,95±0,07	0,68±0,044	18,6±1,38	5,7±0,68
Experiment	180	0,68±0,018	0,71±0,045	0,97±0,088	22,9±2,55	18,4±6,37
Bone Marrow						
Control	30	0,351±0,023	0,715±0,052	0,49±0,03	51,4±11,3	55,8± 8,4
Experiment	15	0,341±0,025	0,604±0,020	0,57±0,05	—	—
"	30	0,322±0,031	0,683±0,029	0,44±0,03	83,0± 5,7	62,5± 4,0
"	90	0,354±0,062	0,351±0,075	0,92±0,05	85,4±16,5	127,2±28,6
Control	180	0,302±0,017	0,595±0,015	0,51±0,04	33,4± 7,8	53,3± 8,6
Experiment	180	0,271±0,033	0,394±0,054	0,70±0,05	87,1±16,8	95,1±11,6

*For specific activity we used the activity of 1 mg P expressed in percent of injected amount of P^{32} per g weight of animal.

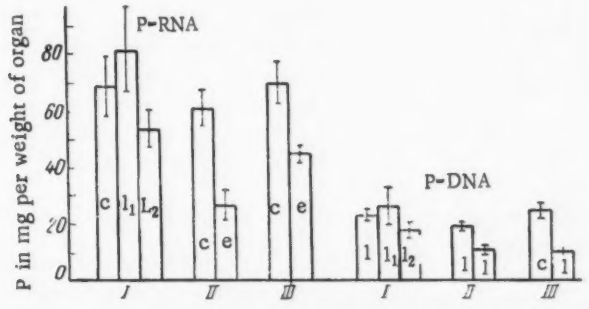

Fig. 1. Content of nucleic acid phosphorus in liver: I) control animals after 30 days (c); experimentals after 15 days (e_1) and after 30 days (e_2); II and III) control animals (c) and experimental animals (e) after 90 and 180 days respectively

organ. The specific activity of P-RNA in the spleen was somewhat increased after 30 and 90 days (by 62 and 45% respectively), but it should be mentioned that the difference between the experimental and control groups after 90 days was statistically insignificant. The DNA specific activity was considerably increased during the whole period of the study.

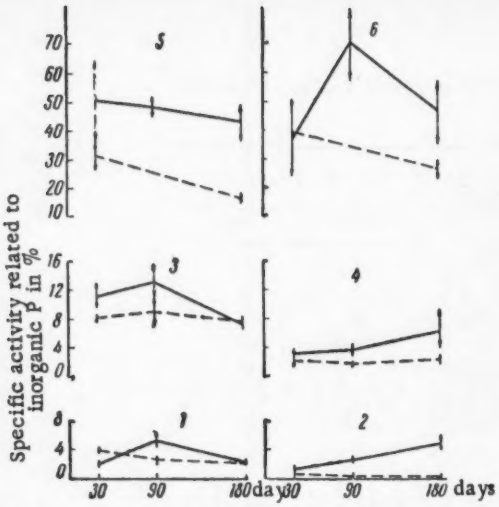

Fig. 2. Relative specific activity of RNA and DNA at various times after injecting plutonium: Dotted lines) controls; solid lines) experimentals; 1) liver RNA; 2) liver DNA; 3) spleen RNA; 4) spleen DNA; 5) bone marrow RNA; 6) bone marrow DNA.

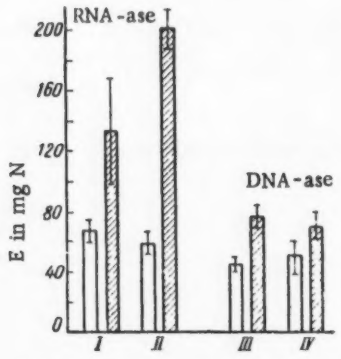

Fig. 3. Depolymerase activity of bone marrow 6 months after injection of plutonium. White columns) control animals; cross-hatched columns) experimental animals: I) acid RNA-ase (pH 5.0; 0.5 mg/ml); II) alkaline RNA-ase (pH 8.2; 2 mg/ml); III) acid DNA-ase (pH 5.0; 2 mg/ml); IV) neutral DNA-ase (pH 7.0; 2 mg/ml).

Analysis of the data on nucleic acids in bone marrow showed the absence of any change in amount of P-RNA during the whole period of the study. The amount of P-DNA decreased by 51% after 3 months and by 33% after 6 months from the injection of Pu. The ratio P-RNA/P-DNA was greatly increased after 2 weeks, 3 and 6 months. The specific activity of RNA was increased during the whole period of study, the greatest increase (2.6 times) occurring at 6 months. The specific activity of DNA after 3 months was 224% and after 6 months 178% of the controls.

The data on dynamics of relative specific activity of nucleic acids in the tissues studied (for the precursor we took inorganic phosphorus, P_i) are shown in Fig. 2. They indicate the increased synthesis of these acids in most of the periods of study. The absence of increased amounts of nucleic acids in bone marrow with their increased synthesis led us to test the activity of the depolymerases. These investigations were carried out on bone marrow tissues 6 months after injection of Pu. The results of these experiments, shown in Fig. 3, clearly indicate that there is a great increase in the activity of DNA-ase and RNA-ase.

In bone marrow tissues we calculated the number of cells per 1 g of tissue. In all the experimental animals we observed a definite decrease in the number of cells (Table 3). In calculation of the averate content of P-RNA per cell we saw plainly that there was an increase of 1.5-2 times in content of RNA during the whole period of the investigation. The average content of P-DNA per cell was quite stable. Some increase was observed only in animals killed after a month.

There is also interest in determining data on the intensity of uptake of P^{32} in the RNA and DNA of bone marrow in the cells. The results show a sharp increase in intensity of uptake of P^{32} in both RNA and DNA of the cells of this tissue which indicates the intensity of renewal of the nucleic acids of bone marrow cells in the experimental animals.

DISCUSSION OF RESULTS

The present investigation has confirmed the existence of a difference in the amount and rate of renewal of nucleic acids in different tissues, apparently chiefly connected with their biological characteristics [1, 13]. Thus, the specific activity of P-DNA in the liver normally averages 1.53, in the spleen 5.6, and in the bone marrow 55.0, that is, the rate of renewal of DNA in bone marrow is about 35 times higher than in the liver. The difference of rate of renewal of RNA is not so great.

As we have already shown previously [12], the total reaction of tissues with long irradiation produced by deposition of plutonium differs appreciably from the reaction after a single application of ionizing radiation. If

TABLE 3

Amount of Nucleic Acids and Intensity of Uptake of P^{32} in Bone Marrow Cells

Group of animals	Period of study, days	Quantity of cells in 1 mg · 10^{-3}	P-RNA in cells, mg · 10^{-10}	P-DNA in cells, mg · 10^{-10}	Intensity of P^{32} uptake in cells, 10^{-3}	
					RNA	DNA
Control	30	9,02±0,60	3,93±0,33	8,08±0,89	2,05±0,55	4,95±1,28
Experimental	15	6,85±0,29	4,96±0,31	8,09±0,59	—	—
"	30	5,83±0,52	5,59±0,61	11,93±1,12	4,82±0,62	7,38±0,37
"	90	3,88±0,64	9,40±0,55	8,82±0,93	9,62±2,17	13,42±2,71
Control	180	7,06±0,42	4,33±0,61	8,48±0,33	1,36±0,38	4,51±0,42
Experimental	180	3,70±0,65	7,42±0,96	10,86±1,55	6,78±1,81	9,89±2,48

with acute irradiation there is a fall in synthesis of nucleic acids in most tissues [1-3, 6], and a decrease in their amount in the early period after irradiation [1, 4, 6], yet with long deposition of plutonium, while there is also a decrease in amount of nucleic acids, their synthesis is markedly raised. A decreased intensity of nucleic acid synthesis was not observed in any period of this study.

A consideration of the relative specific activities of RNA and DNA in the tissues studied (as the precursor we used P_i of the tissues) showed that in the experimental animals in most of the periods there was actually a true increase in nucleic acid synthesis (Fig. 3). It may be that the observed actual differences in change in rate of renewal of nucleic acids in the tissues studied is connected with the difference in strength of dose received by the tissues and the biological characteristics of the cells. Thus, the liver on the average received 350 ber per day, the spleen, 180, and the bone marrow 12 ber. If we consider that the average length of life of a cell in the liver is ten times greater than in bone marrow, then the fact that in bone marrow a small portion of the cells are irradiated, those at the endosteum, makes it possible to understand the observed differences.

Even in 1950 Limperos and Mocher [14] showed that irradiation of rats with doses of 1000-2000 r caused depolymerization of the DNA molecule. According to the results of Kuzin and Budilova [15] the structural viscosity of DNA from the spleen, a day after irradiation with a dose of 1000 r, disappeared completely.

Uspenskaya [16] observed that a dose of Pu which caused death to the animal in the first 2-3 weeks also caused a decrease in structural viscosity of DNA. On the basis of these results we consider it likely that in our experiments there was some depolymerization of DNA, probably due chiefly to rupture of hydrogen bonds.

In the light of the results on increased synthesis of DNA in the tissues of chronically α-particle poisoned tissues, resulting from deposition of Pu, and the probability of some depolymerization of the nucleic acids, we were greatly interested in the conception of Kuzin [17] concerning the hastening effect of DNA depolymerization produced by ionizing radiation on the mitotic activity of the tissue. According to this conception, it would appear that hastening the breakdown of the polynucleotide chain of two reciprocally bound spirals [18] as a result of rupture of hydrogen bonds would automatically cause hastening of DNA synthesis.

We suggest that under the conditions of intensive synthesis of DNA there is not always normal division with formation of two daughter cells, but in a number of cases hastening of synthesis is accompanied by increased cell diameter with appearance of two, three, or even multinuclear cells. Actually, a morphological study of liver preparations carried out by Lemberg showed a considerable number of such multinuclear cells. The increased amount of mitosis and dinuclear cells when β-radioactive substances act on plants has also been observed by Timofeev-Resovskii and Luchnik [19].

The great hastening of DNA synthesis which we observed, however, as mentioned above, did not lead to an increased amount of nucleic acids; on the contrary, their quantity was obviously decreased. The absence of accumulation of nucleic acids and considerable increase in their synthesis can be connected with an increased breakdown of nucleic acids as a result of activation of the enzymatic processes and also as a result of synthesis of incomplete chain acids. A test of the first suggestion about the increased activity of ribo- and deoxyribonuclease in bone marrow tissue actually showed a definite increase in activity of DNA-ase II and alkaline RNA-ase.

The question of the character and biological properties of the newly synthesized DNA cannot be answered

without further experiments. The high ionization density of plutonium α-particles evidently causes rupture in the polynucleotide chain, which may change the biological properties of DNA. The formation of complex compounds of Pu with nucleic acids which was shown by Belyaev in our laboratory, and also by Uspenskaya, working with polonium [12], probably also has an effect on the functional properties of DNA.

Thus, we can establish from our experiments an appreciable hastening of nucleic acid synthesis and an intensification of the processes of splitting, which in morphological studies are related to dystrophic and regenerative processes.

As to the reason for the hastening of DNA synthesis in the tissues under the influence of Pu, we cannot say without further experiments whether this is a primary process which occurs because of a direct hastening of synthesis as a result of rupture of hydrogen bonds in the DNA polymer chain, or is secondary, depending on the increased concentration of breakdown products of the nucleic acids and a compensatory regeneration. Neither can we give a final answer as to whether the decreased number of cells is the result of a chromosome abberation or to a considerable extent depends on the synthesis of incomplete chains of DNA and RNA, damage to synthesis, and the action of specific protein enzymes. A careful experimental analysis of the processes of breakdown and synthesis in plutonium injury will help to solve these questions.

SUMMARY

We have carried out a study of the effect of Pu on the content of nucleic acids and the intensity of uptake of P^{32} in these compounds in liver, bone marrow, and spleen of rabbits 1, 3 and 6 months after injection of the radioactive substance.

When Pu was injected into rabbits in a dose of 7 μcurie/kg, there was a decrease in weight of the liver after 3 and 6 months and a fall in the number of cells in 1 g of bone marrow at all periods of the investigation.

The maximum decrease in content of P-RNA in liver and spleen was observed 3 months after the injection. No change in bone marrow P-RNA was observed. The P-DNA content fell markedly in bone marrow and spleen after 3 months, and in liver after 6 months. The amount of DNA and RNA calculated on the weight of the liver fell more sharply than when calculated on the basis of unit weight of organ. The amount of P-DNA in all the organs after 3 and 6 months was 55 and 46%, and the amount of P-RNA was 56 and 36% of the corresponding results for the control animals.

The specific activity of RNA increased 1.5-2 times in all the tissues in most of the periods of study. The greatest increase in DNA specific activity was in the liver. The activity of bone marrow depolymerases 6 months after injecting plutonium rose; the activity of DNA-aseII by 70%, of acid and alkaline RNA-ase by 96 and 240% respectively.

LITERATURE CITED

[1] G. Hevesy, Radioactive Indicators (IL, Moscow, 1950) [Russian translation].

[2] G. Hevesy, Nature 163, 869 (1949).

[3] K. Abrams, Arch. Biochem. and Biophys. 30, 90 (1951).

[4] C. Lutwak-Mann, Biochem. J. 49, 300, (1951); 52, 356 (1952).

[5] L. S. Kelly, J. D. Hirsch, G. Beach and A. H. Payne, Radiation Res. 2, 490 (1955).

[6] R. E. Libinzon, Biol. Radiats. Med. 3, 57 (1956).

[7] R. E. Libinzon, Proceedings, All-Union Conference on Medical Radiology. Experimental Medical Radiology. p. 130 (Medgiz, Moscow, 1957).

[8] J. N. Davidson, S. H. Frazer and W. S. Huchison, Biochem. J. 49, 311 (1951).

[9] W. C. Schneider and G. H. Hogeboom, J. Biol. Chem. 198, 155 (1952).

[10] K. Morgan, J. Phys. Coll. Chem. 51, 984 (1947).

[11] V. I. Romanovskii, Use of Mathematical Statistics in Experimental Work. (Gostekhizdat, Moscow, 1947).

[12] V. V. Konstantinova and R. E. Libinzon, Vopr. Med. Khim. 5, 339 (1958).

[13] R. M. Smellie, G. F. Humphrey, E. R. M. Kay and J. N. Davidson, Biochem. J. 60, 2, 177 (1955).

[14] G. Limperos and W. A. Mocher, Amer. J. roentgenol. 63, 691 (1950).

[15] A. M. Kuzin and E. V. Budilova, Doklady Akad. Nauk SSSR 98, 961 (1954).

[16] M. S. Uspenskaya, Med. Radiologiya 5, 30 (1958).

[17] A. M. Kuzin, Izv. Akad. Nauk SSSR, Ser. Biol. 3, 273 (1957).

[18] J. Watson and F. Crick, Nature 171, 737 (1953).

[19] N. V. Timofeev-Resovskii and N. V. Luchnik, Proceedings of the All-Union Scientific Technical Conference on Use of Radioactive and Stable Isotopes and Radiation in the National Economy and Science. Radiobiology. p. 258 Izd. AN SSSR (Moscow, 1958).

[20] M. S. Uspenskaya, Biol. Radiats. Med. 2, 68 (1957).

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THE EXISTENCE OF SOME ENZYMATICALLY ACTIVE STATES OF MYOSIN WITH DIFFERENT THERMOSTABILITY

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We showed previously that under the influence of ionizing radiation [1] and also of the sensitizing action of visible light [2] on a solution of myosin, part of the enzyme, while it keeps its adenosinetriphosphatase activity after irradiation, becomes nonhomogeneous. It consists of two components which differ in the size of the energy barrier ($\Delta E^{\neq}$) and rate of reaction of thermal inactivation of the enzymes. One component is inactivated after irradiation just like the unirradiated myosin ($\Delta E^{\neq} = 77$ kcal/mole); the other has an activation barrier of thermal inactivation several times lower ($\Delta E^{\neq} \sim 13$ kcal/mole). Special experiments showed that the value of $\Delta E^{\neq}$ of about 13 kcal/mole was not related to the possibility of aggregation of the irradiated molecule, but characterized some intramolecular reaction. Thus, under the influence of irradiation there was a transformation of part of the myosin molecule into another state characterized by a low activation barrier for thermal inactivation, while preserving enzymatic activity. This effect is not an exclusive peculiarity of myosin. As we showed [1] by treating the numerical data of other authors [3-5] for pepsin and other proteins, this is a general property of various proteins which are quite different in their structure and function. The results of these calculations are given in the last column of Table 1. The phenomenon of radiation "after effect" found in myosin and many other substances evidently depends completely on the transformation of the macromolecule into a similar state [6].

The literature has also described a sharp reduction in the energy barrier of thermal inactivation of proteins with change in acidity of the medium. In a number of proteins in the native state there is some intermediate pH value below which the protein at once would be transformed into another state with a low activation barrier for thermal inactivation. In Table 1 we give values of $\Delta E^{\neq}$ at various pH values for several proteins which were obtained by different authors [7, 8]. We observe the general ability of irradiation and acid media to convert the protein molecules to a state with a low activation barrier, and the grouping of all the values of $\Delta E^{\neq}$ around the values about 13 kcal/mole and about 37 kcal/mole. In the case of egg albumin, for which we know the $\Delta E^{\neq}$ both in acid media and in radiation "aftereffect," the two values actually agree with each other.

In case of similarity or identity of the state into which the protein molecules pass under the influence of irradiation and a definite acidity of the medium, an explanation of the mechanism of this transformation, caused by acids, could throw light on the mechanism of action of radiation and the nature of the "aftereffect." When different states occur, this could explain the specific action of these agents on the protein molecule. Therefore we have carried out the present investigation which consists in studying the effect of acidity of the medium on the kinetics of thermal inactivation of myosin.

METHODS AND RESULTS

A solution of myosin in 0.5 M KCl with a protein concentration of 0.63 mg/ml was thermally inactivated on a water bath with different pH values of the medium. Myosin was obtained by the usual method from rabbit muscle. We measured its adenosinetriphosphatase activity. Samples of the protein were taken at various time intervals after the start of incubation (moment at which the solution reached the temperature of the bath). The

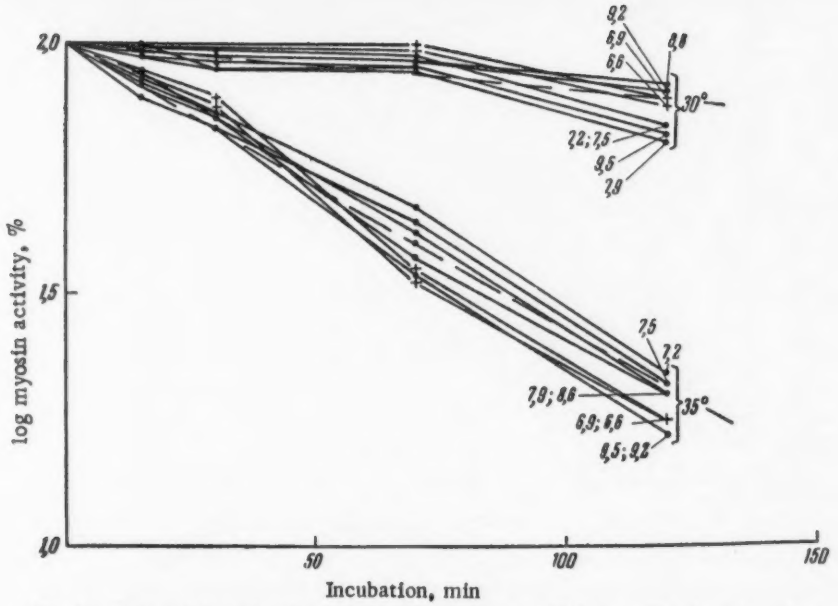

Fig. 1. Thermal inactivation of myosin solutions at different pH values (numbers on the curves) in the range from pH 6.5 to 9.5. Dotted line represents average of data of previous experiments [1] obtained at pH 6.5-7.5.

TABLE 1

Effect of pH and Ionizing Radiation on Energy of Activation of the Thermal Inactivation of Different Proteins ($\Delta E^{\neq}$ in kcal/mole)

Protein	pH value		Effect of ionizing radiation
	pH	ΔE	ΔE of radiation "aftereffect"
Hemoglobin [7]	8,0	77	
	6,8	76	
	5,7	76	
	4,85*	18*	
	4,64*	12*	
	4,08*	12*	
Egg albumin [8]	7,7	134	
	5,0	132	
	3,4	97	
	2,0	48	
	1,62*	37*	
	1,35*	35*	37 (from [3])
Pepsin [8]	6,44	56	13,9—16,7 (from [4])
Trypsin	6,5	40	13 (from [5])
Ptyalin [9]	neutral	123	
	acid	38,5*	
Myosin [1]	6,5—7,5	77	13 (from [1])

*The data relate to the pH range in which the protein goes over to the state with a low activation barrier for thermal inactivation.

required pH value was obtained by dilution of the protein solution with dilute HCl or NaOH, after which the solution was kept for an hour before beginning incubation. The pH was measured with a glass electrode. The pH value of the protein solutions varied from 5.8 to 9.5. In agreement with the data of Lyubimova [10], the activity of myosin (always determined in a borate buffer with pH 9.2) was practically independent of pH in the range mentioned, and fell somewhat (by 0-15%) only on transformation from pH 6.0 to pH 5.8.

The results of the experiments are given in Figs. 1-4, in which are shown on a semilogarithmic scale the curves which characterize the thermal inactivation of myosin at different pH values and temperatures. The general regularity of the thermal inactivation curves is indicated schematically in Fig. 5. As the figures show, the whole pH range used can be divided into three intervals with respect to the character of the thermal inactivation: 1) pH 5.8-6.0; 2) pH 6.0-6.5; 3) pH 6.5-9.5. At the highest pH values (3rd interval) myosin is inactivated exponentially at a rate depending on temperature but not depending on the pH value. From the temperature dependency of myosin inactivation we can determine by the Arrhenius equation the energy of activation of this reaction. In this pH range in accord with the results of our previous study [1] $\Delta E^{\neq}$ = 77 kcal/mole.

In the first interval of pH values (5.8-5.9) the inactivation curve shows a sharp break (Figs. 2-5) which

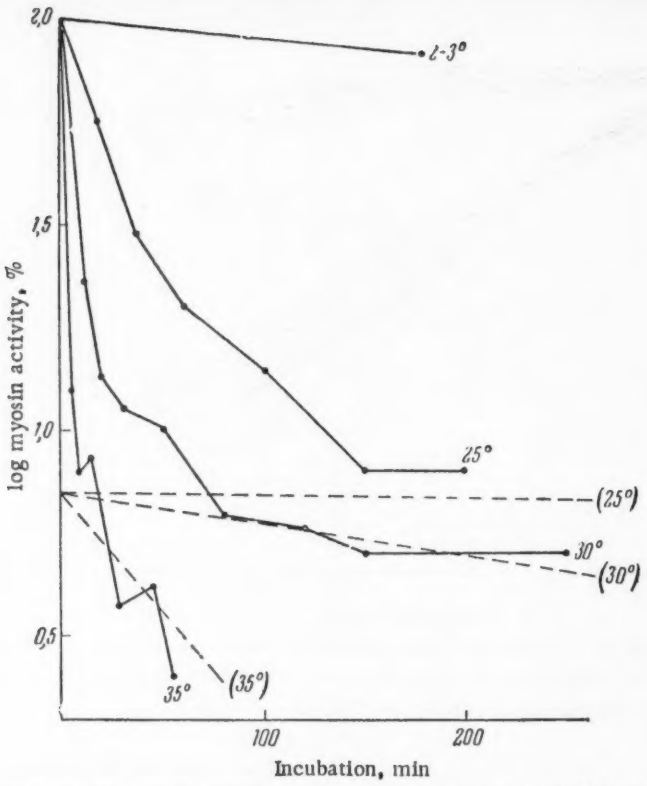

Fig. 2. Thermal inactivation of myosin at pH 5.8 and different incubation temperature (solid line). Dotted line characterizes the slope of the curve of thermal inactivation at higher pH values (6.5-9.5). See Fig. 1.

indicates the existence of at least two protein components which differ greatly in rate of inactivation. This curve appears as the result of placing together two exponents which differ in the index characterizing the rate of inactivation. The quantitative ratio between the two components is such that the greater part of the myosin (70-90%)* occurs in the thermosensitive state. If we analyze the inactivation curves of myosin at these pH values for the two exponents, it is easy to see that the slope of the second, the more slanting of the two at all temperatures, agrees with the slope of the corresponding curves at higher pH.

Hence, the energy barrier of thermal inactivation of this component remains the same as was shown above (77 kcal/mole). This indicates that at pH 5.8-5.9 the more thermostable component is identical with the myosin found in a more alkaline medium. The appearance of the thermolabile component after acidification is connected with some sort of transformation of the protein into a new state which does not differ in enzymatic activity, but reacts differently toward heat.

The data which relate to inactivation of myosin at intermediate pH values (6.0-6.3) are particularly interesting. Here we find a double bend in the inactivation curves (Figs. 3-5). At temperatures of 25-30° inactivation of the protein at first occurs at a rate which is characteristic of the thermostable condition; then part of the protein goes over into the thermosensitive state after a time and under the influence of the incubation. The lower the temperature and the greater the pH value within this "intermediate" range, the less will be the amount of this newly formed thermosensitive component. Moreover, there is apparently an equilibrium

*The break in the curves at pH 5.8-5.9 in different experiments (Figs. 3 and 4) occurs at somewhat different values of residual activity (within the limits 30-10%). This is apparently because of the great dependence of the ratio of both components on the pH and the insufficient accuracy in determining pH by a potentiometer (about 0.05-0.1 pH units).

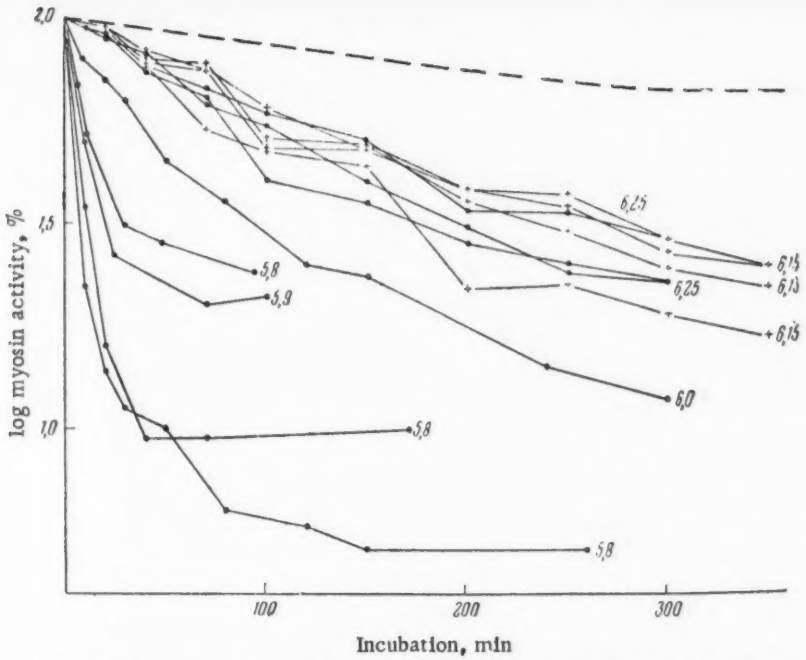

Fig. 3. Thermal inactivation of myosin (30°) at different pH values (figures on the curve) in the pH range 5.8 to 6.25. Dotted lines) inactivation at pH 6.5-9.5 and the same temperature (see Fig. 1).

established between the two components, one of which is inactivated sufficiently rapidly, so that the inactivation curve again assumes the slope of the thermostable component. The higher the temperature and the lower the pH value, the more rapidly will this transformation occur and the shorter will be the part of the curve up to the bend.

Evidently, the character of the transformation is the same at both high and low pH values. but at pH 5.8-5.9 the rate of this change is too great, and the break in the curve is not observed within the limits of accuracy of the method used. At pH values above about 6.5, the transformation from the thermostable to the thermolabile state occurs very slowly and is not shown under conditions of short incubation even at the highest temperature studied (35°). However, in all cases such a transformation evidently occurs. It is shown by the alteration in the curve of thermal inactivation which differs at different pH values and temperatures, and this in turn leads to errors in the determination of the energy barrier, $\Delta E^{\neq}$.

In the pH range 5.8-5.9 at temperatures of 30-35° the transformation of myosin from one state to another occurs quickly and the slope of the curve closely reflects the rate of inactivation of both components. At temperatures of 20-25° the first portion of the inactivation curve is evidently determined simultaneously by two processes: formation of the thermoabile component and its inactivation. Therefore the value for the rate of inactivation of this component obtained from calculation of the slope of the curve may be low which would lead to an alteration of the value of $\Delta E^{\neq}$ toward the higher side.

Nevertheless we determined the value of $\Delta E^{\neq}$ for the thermoabile protein component. As mentioned already, at pH 5.8-5.9 the formation of this component occurs very rapidly and the quantitative ratio of both components is chiefly determined by the pH and is almost independent of the incubation temperature. Actually, with graphical separation of both components and extrapolation of the curves of the thermostable fraction, at the beginning of incubation we observed that the amount of this fraction was practically the same at 25, 30 and 35° (Fig. 2). Under these conditions we can assume that the transformation of the protein at pH 5.8-5.9 occurs before its incubation (in the cold) or at the very beginning, and the curve of loss of activity characterizes only the one process of thermal inactivation of the protein, already composed of two components, in a ratio determined chiefly by the pH value. Graphical extrapolation of the labile component makes it possible to determine

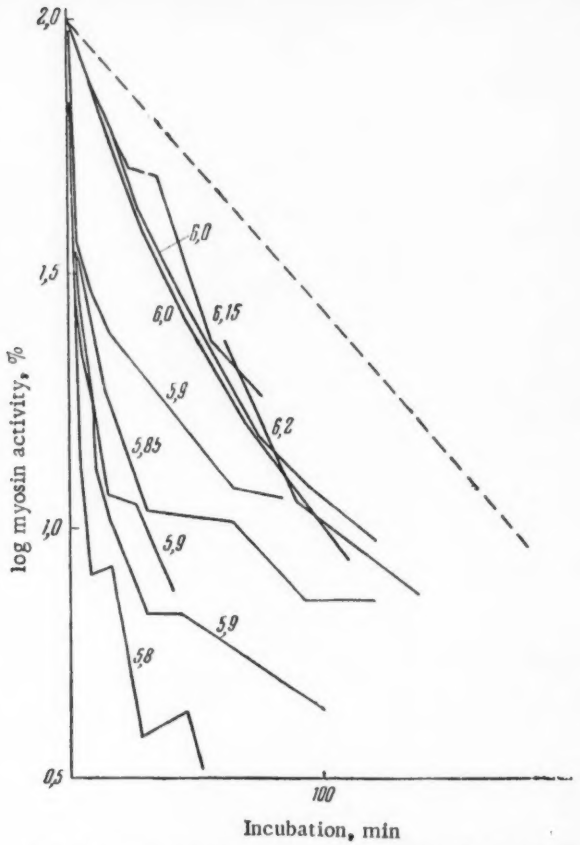

Fig. 4. Thermal inactivation of myosin (35°) at different pH values (figures on the curve) in the pH range from pH 5.8 to 6.2. Dotted line - inactivation at pH 6.5-9.5 at the same temperature (see Fig. 1).

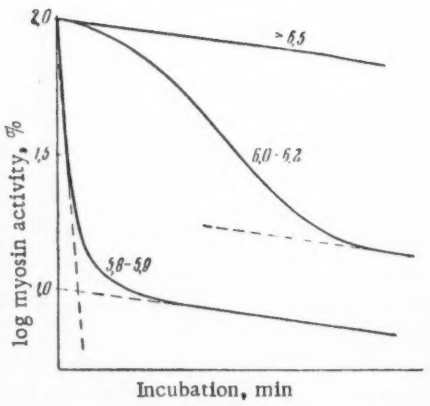

Fig. 5. Schematic representation of the curves of thermal inactivation of myosin at 30° and different pH values (figures on the curves). Dotted line) extension of the two components.

from the temperature relations of its inactivation the value of $\Delta E^{\neq}$ (Fig. 6, Table 2). As the figure shows, the rate of inactivation of this component is ten times greater but the energy barrier of thermal inactivation (55 ± 3 kcal/mole) differs sharply from its value for the intact protein in neutral and alkaline medium and also from the value which is characteristic for the radiation "aftereffect" in irradiated myosin (13 kcal/mole). In Table 2 we give the corresponding values for entropy and free energy of activation (at 25°) calculated from the theory of absolute reaction rates [11].

We should observe that the thermostable fraction, in its turn, is perhaps nonhomogeneous and contains still more thermolabile components. Preliminary results in favor of this assumption indicate from two of our experiments a break in the curve of thermal inactivation of the thermolabile fraction itself (that is, even after calculations to remove the thermostable component).

Probably when the pH of the medium is further lowered to a greater extent than in our experiments there will be

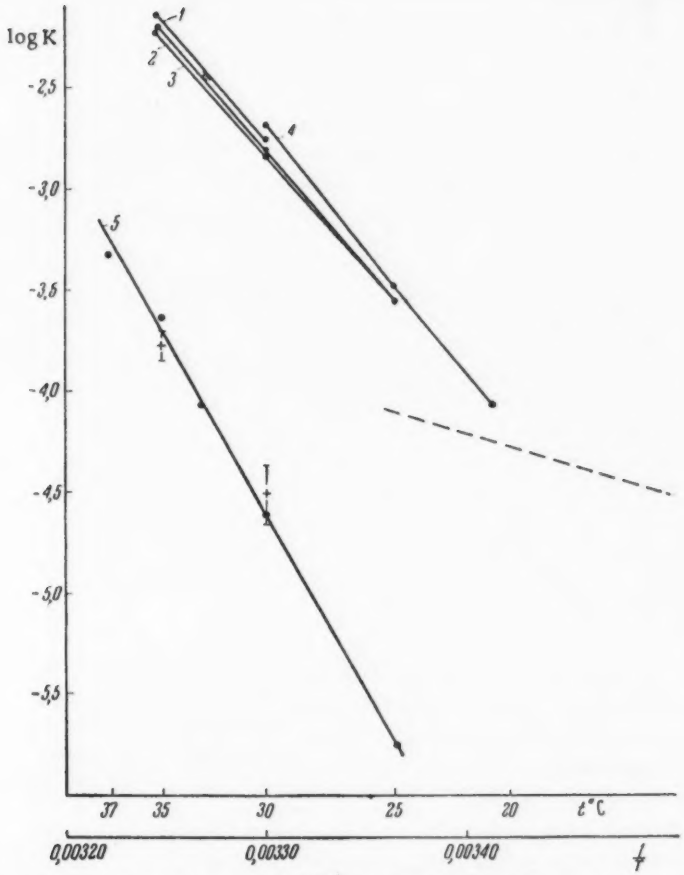

Fig. 6. Determination of $\Delta E^{\neq}$: 1-4) thermolabile component at pH 5.8-5.9; 5) myosin at pH 6.5 and above as a result of previous [1] and the present study (crosses indicate results relating to the thermostable component at pH < 6.5). Dotted line represents results relating to the state of myosin in radiation "aftereffect" ($\Delta E^{\neq}$ = 13 kcal/mole). K) rate constant of reaction of thermal inactivation, sec^{-1}.

TABLE 2

Thermodynamic Data for the Heat of Inactivation of Myosin in its Different States

State of myosin undergoing thermal inactivation	$\Delta F^{\neq}$, kcal/mole	$\Delta E^{\neq}$, kcal/mole	$\Delta S^{\neq}$, cal/mole degree
Thermostable	25,4	77	+174
Thermolabile	22,3	55	+109
Radiation "aftereffect"	23,2	13	— 35

successive new transformations and states will occur which will be characterized by other rates and activation barriers of thermal inactivation.

Reversibility of Myosin Transformations

In a special series of experiments we tested the reversibility of the transformation of myosin from the thermostable to the thermolabile state. For this purpose, myosin solutions with pH 6.5-7.0 were acidified to pH 5.8-5.85, after which they were kept in the cold (1 hour). After we had determined the activation at this new pH value, part of the solution was submitted to thermal inactivation, and another part was again made alkaline, to pH 7.0. A test showed that acidification followed by alkalinization did not cause loss of

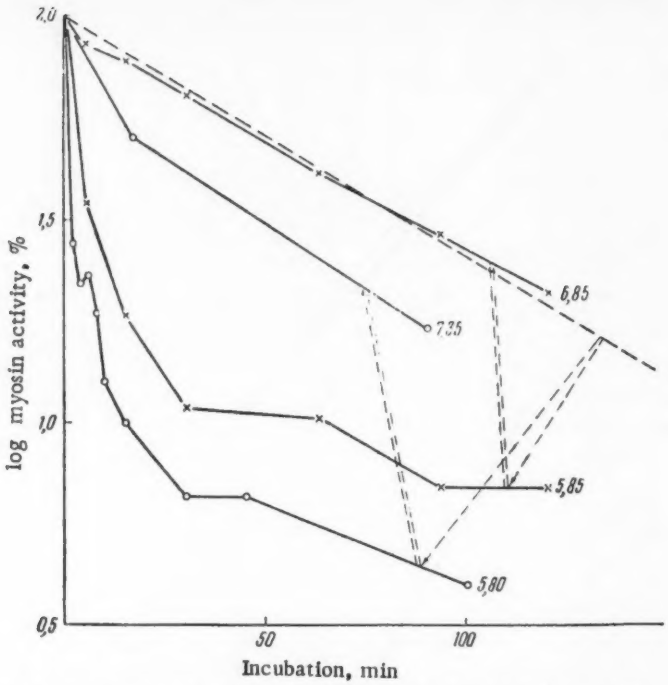

Fig. 7. Reversibility of the transformation of myosin from the thermostable to the thermolabile state. Initial protein (dotted line, pH 6.5) acidified (arrow) and then reversibly alkalinized (double arrow). Thermal inactivation occurred at 35°. pH values shown in figures on the curves. Circles and crosses differentiate the two parallel sets of experiments.

enzyme activity. At the same time, most of the myosin on acidification went over to the thermolabile state, as indicated by the form of the curve of its thermal inactivation. After reverse alkalinization the myosin was thermally inactivated with the normal rate corresponding to the new pH value (7.0). This is illustrated in Fig. 7 in which we give the results of two analogous experiments. The figure shows that this transformation of myosin into the thermolabile state, not accompanied by loss of enzyme activity, is a reversible process within the limits of the investigation using the given changes of pH and temperature.

The full and rapid reversibility found for the myosin transformation indicates, it seems to us, that this change is not the result of a split from the myosin molecule of fragments which retain the enzymatic activity but differ in their thermolability. The reverse combination of such fragments (which should contain 70-90% of all the centers of enzyme activity of the myosin) would be incomplete after alkalinization and would require a definite time.

Thus, the effect which we have described evidently means the transformation into another state of the same myosin molecule, and not the splitting of fragments from it.

We should mention that in the process of inactivation of the thermolabile part of the protein the ratio of both components is not changed in the solution. In the opposite case, as inactivation proceeded all of the remaining protein would be transformed into the thermolabile state, which is not actually found: the quantitative ratio of both components is retained also after inactivation of the thermolabile fraction.

The new, more thermolabile state formed by acidification of the myosin solution has a "looser" structure, resembling the structure of the activated complex formed in the process of thermal inactivation of myosin. This is indicated by the lower values for the heat and entropy of activation in the thermolabile state compared to the ordinary one (Table 2).

Thus, under the influence of acids there is a loosening of the protein structure which cannot destroy its ability to act as an enzyme. Within the known limits, this structural change is reversible. It can be produced by change in pH of the medium,* temperature, and probably by the action of other agents. It is not impossible that in such transformations other properties of the protein molecule besides thermosensitivity are changed. In particular, there must be a study of which bonds are involved in the transformation with structural change of the protein which was found by Doty [12], and also in the reversible transformation of egg albumin under the influence of acid and alkali recently observed by Belitser and Saenko [13]. In their work, egg albumin in strongly acid or strongly alkaline media was reversibly transformed into some state which was characterized by different specific rotation as compared to the usual conditions.

The general decrease in the values of $\Delta E^{\neq}$ in thermal denaturation of different proteins at high pH values found by a number of authors [7, 8] is apparently also explained by gradual formation of a more thermolabile component when the protein solutions are acidified. The fact that in studies carried out with other proteins a direct transformation of the protein from one state to another was not observed can be explained. Actually, to observe the simultaneous presence in the solution of both myosin components was possible only in a sufficiently sharp "transition" range of pH and temperature. Outside this range the chief mass of the protein occurred in one of these states and the amount of the other was very small. The accuracy of determination of enzymatic activity in work in which the thermal inactivation of different proteins was studied was small and the incubation was usually stopped at 10-20% residual activity.

Very recently two studies have appeared which in our opinion have a direct relation to the phenomena which we have observed in myosin, and thus to its generality. Cartwright and Lauffer [14] studied the effect of temperature on the toxin of Botulinum A. They showed that the thermal inactivation curve of the toxin solutions, as in our case, gave a break which indicated a nonhomogeneous state. This was observed only on incubation at 50-60° and did not appear at lower temperature (40°). The rate of inactivation of both components depended on temperature, and in the conditions of their experiment, more than 95% of the toxin was converted to the thermolabile fraction. The authors themselves were inclined to assume the dissociation of the toxin molecule under the influence of heat into two active parts which had different thermosensitivities. However, this conclusion was based on various indirect reasons. The question of the possibility of reversibility of this transformation was not studied by the authors, unfortunately.

In our opinion, the most complete and interesting data were obtained in the work of Kaplan [15], who studied the thermal inactivation of the virus of smallpox vaccine. In distinction to work with enzymes, work with viruses has the possibility of being carried out with many orders of values. The results of Kaplan's experiments led to the conclusion that smallpox vaccine virus is inactivated as if it were composed of two components. Their quantitative ratio depended on temperature, in connection with which the author expressed the opinion that his virus consisted of a main mass of a thermolabile character and a small amount of a substance which had a variable sensitivity. A satisfactory explanation of the nature of this inhomogeneity has not yet appeared.

We call attention to the full similarity of the results obtained in the studies we have cited on viruses and toxins with our work on an individual protein, myosin. In the case of myosin, the possibility of a nonhomogeneous starting protein is excluded by the fact that we have followed the dynamics of the transformation from one state to the other and the reversibility of this condition.

The observed behavior of viruses on thermal inactivation can be explained if we assume that under the action of heat there is a transformation of the nucleoprotein of the virus into a thermolabile state like that which we found under definite conditions for myosin.

Comparison of the Action of Radiation and Change in pH

The results which have been given indicate that the native state of myosin with a lowered activation barrier for thermal inactivation occurs under the action of acids and ionizing radiation, but these are not

*We have not studied the behavior of myosin in the alkaline range systematically, but test experiments show that also here (pH about 10.1) a thermolabile component is formed. Thermal inactivation of myosin in an alkaline medium was studied by Lyubimova [10]. Her experimental material can also serve as an indication that in the alkaline range there is formation of a thermolabile component of the protein.

identical with each other. They do not differ only in the value of $\Delta E^{\neq}$ of thermal inactivation. In distinction from the case of inactivation in the acid medium, the reaction of radiation "aftereffect" is characterized by a negative value for the entropy of activation. This indicates that the thermal inactivation of irradiated but enzymatically active molecules occurs through a stage of formation of an activated complex which exists in a more oriented, more "directed" state than the original protein. The reverse effect is found in the case of the precursor of the acid medium, where activation occurs through a "looser" molecule.

Thus, the initial state of myosin itself and the path of its activation in thermal inactivation is different in the two cases. They differ also in the reversibility of the transformation from the thermostable to the thermolabile state. Actually, if we consider the possibility of the occurrence in the track of the ionizing particles during irradiation, as a result of radiolysis, of local changes in the pH of the solution, we can try to explain by the same mechanism the general ability of radiation and acidity of the medium to form a thermolabile state of the macromolecule. However, as we showed above, the transformation into such a state is reversible with change in pH.

In our experiments, after irradiation by γ-rays in doses which caused even full inactivation of myosin, no apparent change in pH occurred. Therefore, if there were a local change of pH in the solution, the final state of the myosin should not be different from the original before irradiation. This was not the case. Hence, the transformation of the myosin molecule into the thermolabile state under the influence of radiation is an irreversible reaction.

Thus, our results indicate a sharp specificity in the action of radiation on the myosin molecule.

SUMMARY

By change in the acidity of the solution we have found the formation of several (at least two) enzymatically active states of myosin which differ from each other in thermal sensitivity. The rate of thermal inactivation of myosin in the more thermolabile state is on the order of twice as great, but the energy barrier ($\Delta E^{\neq}$ = = 55 kcal/mole) is less than in the thermostable state (ΔE = 77 kcal/mole).

The more thermolabile component is formed from the thermostable one, as can be observed at temperatures of 20-30° and decrease in pH to 6.3-5.8. This transformation is reversible, but the quantitative ratio of both components depends on pH and on the temperature of the medium. The thermolabile, enzymatically active state of myosin which occurs at definite pH changes and that which occurs in irradiation differ sharply from each other in their properties.

Analysis of the literature suggests the general character of the transformation which myosin undergoes in common with a number of other proteins.

In conclusion, the authors express thanks to V. Yu. Urbakh for discussion of the results obtained and for a number of valuable comments.

LITERATURE CITED

[1] L. Kh. Éidus and E. É. Ganassi, Biofizika 4, 215 (1959).

[2] L. Kh. Éidus and N. V. Kondakova, Biofizika 4, 378 (1959).

[3] J. H. Clark, J. Gen. Physiol. 19, 199 (1935).

[4] R. S. Anderson, Brit. J. Radiol. 27, 56 (1954).

[5] M. R. Mac Donald, Brit. J. Radiol. 27, 62 (1954).

[6] L. Kh. Éidus, Biofizika 1, 544 (1956).

[7] H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, Chem. Rev. 34, 157 (1954).

[8] H. Eyring and A. S. Stearn, Chem. Revs. 24, 253 (1939).

[9] V. Z. Henriques, Physiol. Chem. 224, 129 (1954).

[10] M. N. Lyubimova, Enzymatic Properties of Myosin from Cross Striated Muscle. Dissertation (Moscow, 1957).

[11] S. Glasstone, K. Leidler and G. Eyring, Theory of the Absolute Rate of Reactions (IL, Moscow, 1948) [Russian translation].

[12] P. Doty, Col. Czechoslovak Chem. Communic. (spec. issue) 22 (1957).

[13] V. A. Belitser and T. V. Saenko, Biokhimiya 22, 274 (1957).

[14] T. E. Cartwright and M. A. Lauffer, Proc. Soc. Exptl. Biol. and Med. 98, 327 (1958).

[15] C. Kaplan, J. Gen. Microbiol. 18, 58 (1958).

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IMMUNO-ELECTROPHORETIC ANALYSIS OF BLOOD SERUM PROTEINS IN RADIATION SICKNESS

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E. M. Belyaeva

The study of the protein fraction of blood serum in radiation sickness has been carried out by a large number of authors [1-8] who have established a lowered content of the albumin fraction and an increased amount of α- and β-globulins, especially the α-globulins. These changes have been shown by the ordinary methods of electrophoresis. The electrophoretic method in the form in which it has been used until recently has been able to show only a small number of protein fractions, from 5 to 7.

In recent times a perfected method of electrophoresis has been described: two phase electrophoresis [9] which has shown 21 protein fractions in blood serum, and immuno-electrophoresis which has shown 19 fractions [10- 12].

We have carried out an investigation of the protein fraction of blood serum of rats after total action of x-rays in a dose of 600 r by the immuno-electrophoretic method of Grabar [10-12].

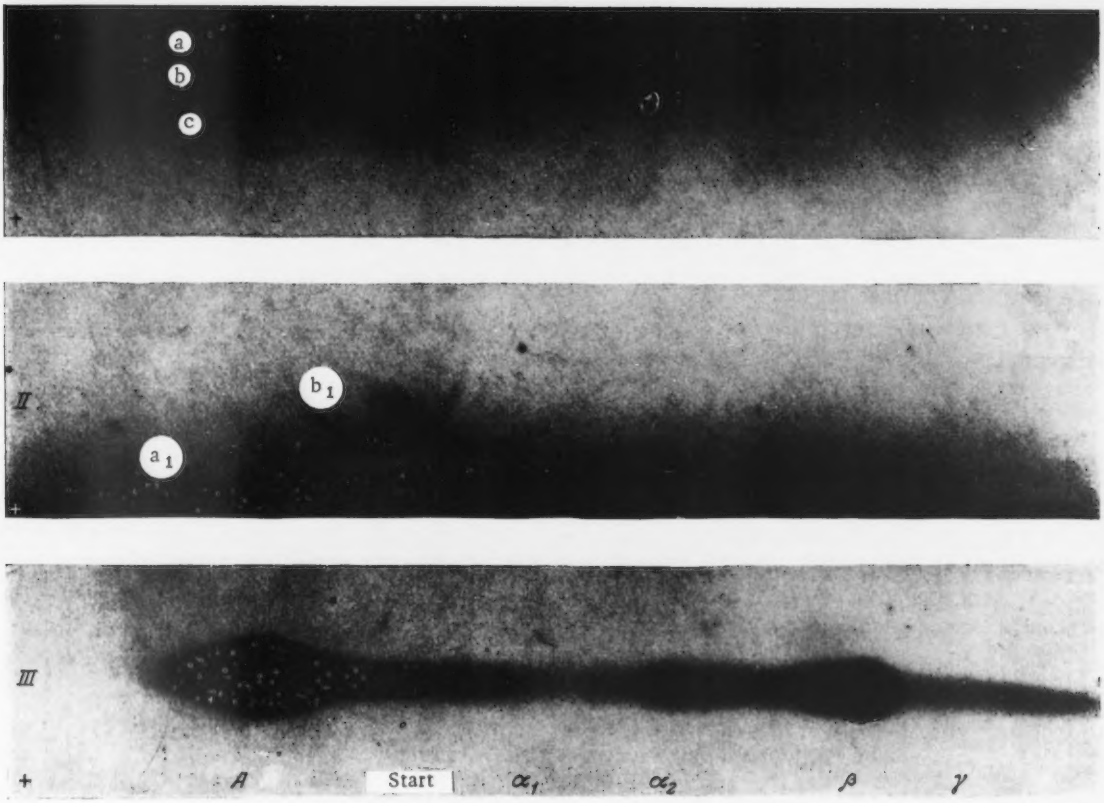
METHODS

In the modification of electrophoresis on agar which was used,we carried it out on a glass slide placed on a special bridge with a rim. In order to obtain the starting cell for the study of serum, and also the groove for the antiserum we placed in the melted agar on the glass two forms connected with each other by a strip. We thus had the possibility of obtaining each time a mould with the standard cell of the same dimensions and spaced at the same distance from the groove with the antiserum. After we had filled the starting cell with the mixture of serum and agar and cooled the mixture, we cut off the upper layer of the whole mould along the upper layer of the cooled contents of the starting cell at the level of the rim with a razor blade. The remaining lower layer always had a standard thickness with a smooth surface and contained at the point of the starting cell a piece of the solidified mixture of serum and agar of a cylindrical form. In one cell we placed the serum of control rats, and in another the serum of irradiated rats.

The rate were irradiated with x-rays in an RUM-3 apparatus with one total dose at 180 kv, 15 ma current strength, with a filter of 0.5 mm Cu and 1 mm Al, at a skin focus distance of 40 cm and a strength of dose 30 r/min. The serum for investigation was taken from rats irradiated with a dose of 600 r. For immunization of rabbits we used serum from rats irradiated with a dose of 600 and 800 r. Blood was taken from the rats on the fifth day after irradiation with 600 r and on the third day with a dose of 800 r. We ran 16 experiments.

The first eight pairs of electrophoretograms were run with antiserum of two rabbits immunized with whole serum of rats irradiated with 800 r. Immunization of the rabbits was carried out as follows: 9 intravenous injections with 3 day intervals with 0.05 ml of serum in 1 ml of physiological saline; after 1 and 4 months, one injection of undiluted serum in amounts corresponding to 0.5 and 1 ml.

The next eight pairs of electrophoretograms were run with antiserum of two rabbits immunized with serum from rats which had received 600 r. Immunization was carried out as follows: over two weeks, 5 injections with 0.5 ml of rat serum in 1 ml of physiological saline; after one month, a single injection of 1.0 ml of undiluted serum. In all the series, the blood from the rabbits for supplying the antiserum was taken eight days after the last injection.



Change in the immunoelectrophoretogram of blood serum proteins in radiation sickness. 1) Immunoelectrophoretogram of control rats; II) irradiated rats (600 r, 5 days after irradiation); a, b, c and a_1, b_1) arcs of precipitate in albumin field of the electrophoretograms of sera of control and irradiated rats. Arc b_1 is less shifted to the anode than arc c. Both electrophoretograms are developed with antisera of rabbits immunized with whole serum of irradiated rats; III) electrophoretogram of proteins from the blood serum of intact rats stained without immunodevelopment. A) Albumins; α_1, α_2, β and γ-individual globulin fractions.

The antiserum obtained under these conditions of immunization showed a small number of precipitation arcs, especially in the albumin field where there were only three arcs instead of the nine produced by "strong" antiserum obtained after more intense immunization. However, the effect of this "weak" antiserum was to differentiate more clearly the difference between the immunoelectrophoretograms of the sera of irradiated and unirradiated rats.

In 13 cases out of 16 immunoelectrophoretograms the serum of irradiated rats differed from the immunoelectrophoretograms of the serum intact rats. The difference consisted in the disappearance in the albumin field of a middle are in the immunoelectrophoretograms of the serum of irradiated rats (figure). The arc which lay near the groove and the arc lying near the starting cell did not disappear. In five cases the middle arc which was not visible without staining appeared after staining and then the immunoelectrophoretogram became like the immunoelectrophoretogram of the serum of healthy rats. This is evidently because the antigen corresponding to this precipitation arc does not disappear entirely, but is greatly decreased in amount so that only a little precipitate appears and cannot be seen without staining. This fact which we have established indicates the disappearance of one protein in the albumin fraction or a great decrease in its amount.

In some cases, the arc in the albumin field near the starting cell was less shifted toward the anode in the immunoelectrophoretogram from the serum of irradiated rats. This indicates that in some cases in radiation sickness there is a change in the electrophoretic properties of the corresponding protein of this fraction.

No new, unusual arcs appeared in the electrophoretogram of the serum of irradiated rats.

SUMMARY

Our experiments have shown that in irradiated animals the content of different proteins in the albumin fraction is decreased unequally, and in particular we have shown the disappearance or sharp decrease in amount of one of these proteins. The electrophoretic mobility of some serum proteins changes in radiation sickness.

LITERATURE CITED

[1] J. A. Müntz, E. S. G. Barron and C. L. Prosser, Arch. Bioch. 23, 434 (1949).

[2] L. Hempelmann, G. Lisco and D. Hofmann, Acute Radiation Sickness (IL, Moscow, 1954) [Russian translation].

[3] H. J. Kohn, Amer. J. Physiol. 162, 703 (1950).

[4] V. S. Balabukha, in the book: Metabolism in Radiation Sickness [Medgiz, Moscow, 1956) p. 105.

[5] V. D. Blokhina, Metabolism in Radiation Sickness (Medgiz, Moscow 1956) p. 102.

[6] J. L. Zaets, in the book: Metabolism in Radiation Sickness, by L. I. Ivanov, V.S. Balabukha and others (Medgiz, Moscow, 1956) p. 109.

[7] A. K. Gus'kova and G. D. Baisogolov, Speeches of the Soviet Delegation to International Conference on Forceful Uses of Atomic Energy, Geneva, Izd. AN SSSR.(Moscow, 1955).

[8] R. Bauer, S. Piller and G. Schneider, Strahlentherapie 100, 16 (1956).

[9] O. Smities, Nature 177, 1033 (1956).

[10] P. Grabar and C. A. Williams, Biochim. et biophys. acta 10, 193 (1953).

[11] P. Grabar and C. A. Willians, Biochim. et biophys. acta 17, 67 (1955).

[12] P. Grabar, Biokhimiya 22, 49 (1957).

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THE SYNTHESIS OF TRYPTOPHAN FROM INDOLEPYRUVIC ACID IN PLANTS

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We have shown earlier that tryptophan is formed from indole or anthranilic acid when they are infiltrated by vacuum into pea seedlings [1] and the synthesis of tryptophan is strengthened if serine is introduced simultaneously. In the present work we have studied the formation of tryptophan from what is probably its closest amino-nitrogen free precursor, β-3-indolepyruvic acid (IP). IP was first synthesized by Ellinger and Matsuoka [2] who considered it to be the first product in the transformation of tryptophan. It was later shown that IP can serve as a precursor for tryptophan in microorganisms [3] and in model systems where IP reacts by transamination with various amino acids [4, 5] and kynurenine [6]. The formation of tryptophan from IP can occur so intensely that Sakurai could propose a method for enzymatic preparation of L-tryptophan from IP [7].

In plants, IP is evidently a precursor of both tryptophan and indoleacetic acid. There are no reports in the literature of the formation of tryptophan from IP in plant organisms.

EXPERIMENTAL

Methods

The synthesis of IP was carried out by the method of Ellinger and Matsuoka* and by the method of Bentley and co-workers [8]. The latter method gave markedly higher yields of IP which was identified by the point of beginning decomposition at 190°, spectrophotometrically (absorption maxima at 328 and 232 mμ in 96% ethanol), and chromatographically (solvent: acetic acid-water in the ratio 1:3 with development by p-dimethylaminobenzaldehyde, a blue spot with a green edge). Judging by the value of the molar extinction at 328 mμ the purity of the preparation obtained by the method of Ellinger and Matsuoka was 94%; the purity of IP obtained by the method of Bentley as judged by the same method was nearly 100%.

For experiments with whole plants we used the green part of wheat seedlings without endosperm and roots, and the green part of pea seedlings without cotyledons and roots. The length of the seedlings was 6-7 cm. The solutions being studied were introduced into the seedlings by forcing in. After the introduction, 3 g of seedlings were fixed with four times the volume of freshly distilled boiling 96% ethanol. The extraction was repeated twice, the extracts were combined and filtered hot. The extracts were evaporated, first in a stream of dry air and then in a vacuum desiccator over H_2SO_4 or $CaCl_2$. The dry residue was dissolved in 10% isopropanol, acidified with hydrochloric acid to pH 3, and the volume of each sample was brought to 1 ml. Ether extraction was then carried out. In the ether, according to our results and the literature data [3], were found pigments, IP, and acid products of breakdown of IP, including indoleacetic acid, but the tryptophan remained in the water layer. After removal of the ether, the water layer was neutralized for determination of tryptophan.

For experiments with whole extracts we used green seedlings of wheat, barley, or peas with length 6-7 cm which were ground with a small amount of washed quartz sand in the cold (the mortar was cooled with a mixture of ice and salt) with an equal weight of distilled water or phosphate buffer. The resulting paste was pressed through a double layer of sterile gauze. The liquid thus obtained was treated with the reaction components,

*Recrystallization of IP obtained by the method of Matsuoka was carried out as described by Bentley et al. [8].

TABLE 1

Formation of Tryptophan from IP in Extracts from Seedlings. Experimental Conditions: extract 5 ml, IP 10 mg (0.046 mmoles), $(NH_4)_2HPO_4$ 13.2 mg (0.1 mmole), glutamic acid 29 mg (0.2 mmole), ATP 15 mg, incubation 4 hours. In the first two experiments IP, glutamic acid, and ATP were neutralized before adding the extract, using K_2CO_3, and in the two following experiments, neutralization was carried out with KOH or NH_4OH

Extract from seedling of	Composition of sample	pH	Content of tryptophan in experimental mixture			Effect of ATP	
			τ	Δτ	Δ%	Δτ	Δ%
Pea	Extract without additions (in water)	6,2	53				
	The same + K-IP + $(NH_4)_2HPO_4$		286	233	440		
	" + K-IP + $(NH_4)_2HPO_4$ + + K-ATP		400	347	655	114	49
	" + K-IP + K-glutamate		666	613	1157	—	—
	" + K-IP + K-glutamate + + K-ATP		531	478	902	—135	—22
Wheat	Extract without additions (in phosphate buffer)	7,5	60	—	—	—	—
	The same + K-IP		190	130	217	—	—
	" + K-IP + $(NH_4)_2HPO_4$		200	140	233	—	—
	+ K-IP + $(NH_4)_2HPO_4$ + + K-ATP		310	250	417	110	79
	" + K-IP + K-glutamate		540	480	800	—	—
	" + K-IP + K-glutamate + + K-ATP		400	340	567	—140	—29
Barley	Extract without additions (in water	6,2	193	—	—	—	—
	The same + K-ATP		186	—7	—3,6	—	—
	" + K-IP		253	60	31	—	—
	" + NH_4-IP		253	60	31	—	—
	" + K-IP + K-ATP		306	113	58,5	53	89
	" + NH_4-IP + NH_4-ATP		333	140	72,5	80	133
Pea	Extract without additions (in water)	6,2	113	—	—	—	—
	The same + K-ATP		127	14	12	—	—
	" + NH_4-ATP		120	7	6	—	—
	" + K-IP		234	121	107	—	—
	" + NH_4-IP		227	114	101	—	—
	" + K-IP + K-ATP		495	382	338	261	215
	" + NH_4-IP + NH_4-ATP		320	207	183	93	82

neutralized to pH 7.0, and incubated for 4 hours. After this the sample was fixed with boiling ethanol and further treated as described for the whole seedlings.

Tryptophan was determined by the chromatographic method which we described previously [1] on "Leningrad slow" or "Leningrad fast" paper with p-dimethylaminobenzaldehyde (p-DAB) as the developer. As the solvent we used butanol saturated with phosphate buffer, pH 4.5 or pH 6.2, and paper saturated with the same buffer. Under these conditions the tryptophan spot even with high salt concentrations in the extract studied was not diffuse in chromatography, and had R_f 0.20-0.25. Indoleacetic acid under the same conditions of chromatography had R_f 0.78. The mean square deviation in the chromatographic determination run with the aid of p-DAB on pure solutions of tryptophan was ± 1%. In some cases tryptophan, like other amino acids, was determined by the ninhydrin method described below. This method is considerably less accurate for tryptophan, but still gives satisfactory agreement with the first method.

The determination of all the other amino acids was carried out by the method described by Kretovich and Uspenskaya [9] with single phase descending chromatography in buffered phenol (phosphate buffer, pH 11.5) or buffered cresol (phosphate buffer, pH 6.2) on paper soaked in the corresponding buffer.

TABLE 2

Effect of Glutamic Acid and Isoniazide on the Formation of Tryptophan from IP. Experimental conditions: extract 5 ml; IP 10 mg (0.046 mmole); $(NH_4)_2HPO_4$ 13.2 mg (0.1 mmole); glutamic acid 29 mg (0.2 mmole); ATP 15 mg; isoniazide 5 mg. IP, glutamic acid, and ATP neutralized with K_2CO_3 before addition of the extract. Incubation lasted 4 hours.

Extract from seedlings of	Composition of sample	pH	Tryptophan content in experimental mixture			Effect of isoniazide	
			T	ΔT	Δ%	ΔT	Δ%
Barley	Extract (in water)	6,2	266				
	The same + K-IP + $(NH_4)_2HPO_4$ +		680	414	156		
	" + K-IP + $(NH_4)_2HPO_4$ + + K-ATP + isoniazide		720	454	170	40	9,7
	" + K-IP + K-glutamate		1340	1074	404	—	—
	" + K-IP + K-glutamate + + isoniazide		800	534	201	—540	—50
Wheat	Extract (in phosphate buffer)	7,8	100	—	—	—	—
	The same + K-glutamate		100	0	0	—	—
	" + K-glutamate + isoniazide		100	0	0	0	0
	" + K-IP + K-ATP		560	460	460	—	—
	" + K-IP + K-ATP + izoniazide		480	380	380	—80	—17
	" + K-IP + K-ATP + $(NH_4)_2HPO_4$		560	460	460	—	—
	" + K-IP + K-glutamate		960	860	860	—	—
	" + K-IP + K-glutamate + + isoniazide		580	480	480	—380	—44

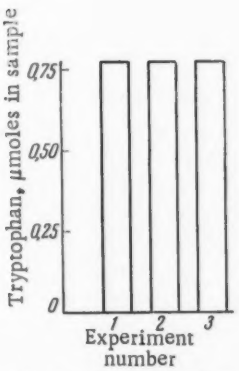

Fig. 1. Synthesis of tryptophan from a boiled solution of IP in extracts from barley seedlings. Experimental conditions: 1) 5 ml of extract without IP; 2) 5 ml of extract + 10 ml of boiled IP + 13.2 mg $(NH_4)_2HPO_4$ + + 15 mg ATP; 3) 5 ml of extract + 10 mg of boiled IP + 34 mg monosodium glutamate.

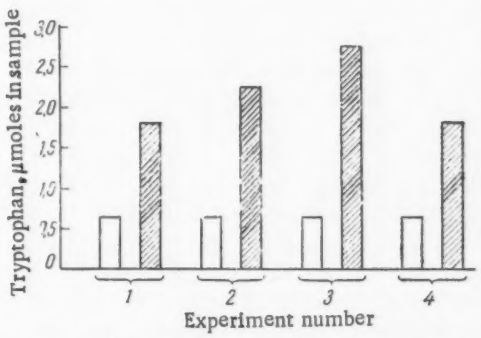

Fig. 2. Effect of concentration of ATP on formation of tryptophan from IP in extracts from green barley seedlings. Experimental conditions: 5 ml seedling extract; 10 mg IP; IP and ATP neutralized by K_2CO_3, final pH 7.7, incubation 4 hours: 1) without ATP; 2) 5 mg ATP added; 3) 15 mg ATP added; 4) 45 mg ATP added. White columns) without IP; crosshatched columns) 10 mg IP added.

RESULTS

Experiments with Extracts from Plants

The experiments were carried out on extracts from seedlings of peas, wheat and barley. The synthesis of tryptophan was found when the extracts were incubated with IP alone, neutralized by K_2CO_3, without any other addition (Table 1).

TABLE 3

Synthesis of Tryptophan in Wheat and Pea Seedlings in the Dark and in the Light. The IP in water in the first and second experiments was neutralized once before beginning infiltration with KOH to pH 8. In the third and fourth experiments the neutralization to pH 8.0 was twice repeated during the infiltration. Final IP concentration in solution 0.05 M

Experiment No.	Seedling	Infiltrated solution	Length of infiltration, hours	Tryptophan content					
				in light			in the dark		
				μmole/g wet wt. of seedlings	Δ in μmoles	Δ, %	μmole/g wet wt. of seedlings	Δ in μmoles	Δ, %
1	Wheat	H_2O	4,5	0,120	—	—	0,120	—	—
		K-IP		0,500	0,380	316	0,270	0,150	125
2	Pea	H_2O	6,0	0,17	—	—	0,20	—	—
		K-IP		2,40	2,23	1310	1,17	0,97	486
3	Wheat	H_2O	5,5	0,150	—	—	0,150	—	—
		K-IP		0,673	0,523	350	0,390	0,240	160
		NH_4-IP		0,743	0,593	396	0,543	0,393	262
4	Wheat	H_2O	10				0,413	—	—
		K-IP					1,148	0,735	177
		NH_4-IP					1,267	0,854	206

However, as is known from the literature [8], when IP is incubated in an aqueous medium it undergoes a gradual partial decomposition. We confirmed this and then had to show that in our experiments the synthesis of tryptophan actually occurs from IP and not from the products of its decomposition. For this purpose, before adding the plant extract we incubated two samples with IP neutralized by K_2CO_3 for 6 hours in water, heating from time to time on the boiling water bath. Results of spectrophotometric determination at 330 mμ showed that after this treatment one sample still contained 10% and the other 6% of the added IP. Chromatographic analysis (solvent: acetic acid-water in the ratio 1:3, ascending chromatogram) of the incubated sample with development by p-DAB gave 5 spots. The weakly colored spot with Rf 0.43, judging by the R_f value and the color with p-DAB, was IP. Clearest and largest was the spot with R_f 0.79 which was identified by R_f and color as indoleacetic acid. Judging from the results of quantitative chromatographic determination, the amount of indoleacetic acid formed was 30% of the taken IP. Thus, starting from the results of chromatographic and spectrophotometric measurements, IP is decomposed to a considerable extent under rather severe conditions with the formation of indoleacetic acid as the chief product. The IP preparation destroyed by such treatment was treated in one case with $(NH_4)_2HPO_4$ and ATP, and in the other by monosodium glutamate and then with a freshly prepared extract of barley seedlings. As the results show (Fig. 1), under these conditions there is no tryptophan formation from the breakdown products of IP. Hence, tryptophan in our experiments was actually formed from IP, and not from its decomposition products. We also showed that glutamic acid as the potassium salt stimulates formation of tryptophan from IP. Table 1 shows that potassium glutamate increases the formation of tryptophan from IP 2-3 fold compared to samples to which only IP was added without glutamic acid.

In order to explain the share of the transamination reaction in the formation of tryptophan from IP in the presence of added glutamic acid, we ran experiments with the addition of isonicotinic acid hydrazide (isoniazide) to the reaction mixture as an inhibitor of the pyridoxal enzyme. Isoniazide was first incubated for 30 min with the extracts and then the other components of the reaction were added.

Table 2 shows that isoniazide decreases to a considerable degree the stimulating action of glutamic acid. However, isoniazide does not suppress the formation of tryptophan from IP completely, and in samples to which glutamic acid was not added but ATP was it showed no action or a very slight one. Judging by the inhibiting action of isoniazide, transamination has a definite part in the formation of tryptophan from IP in the extracts only when glutamic acid is added. When ATP is added to the extracts, the specific part of transamination in the synthesis of tryptophan is evidently slight.

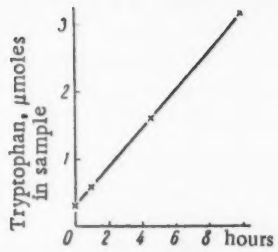

Fig. 3. Effect of duration of infiltration on tryptophan synthesis from IP in wheat seedlings in the light. IP was infiltrated in the form of a 0.5 M solution of the potassium salt.

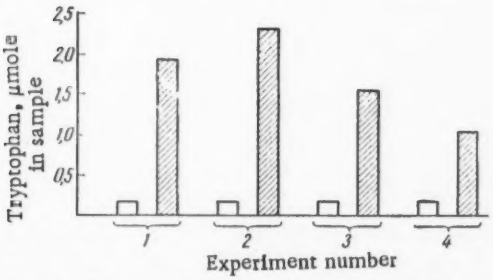

Fig. 4. Effect of different concentrations of ammonium phosphate on the formation of tryptophan from IP in wheat seedlings IP infiltrated as a 0.5 M solution of the potassium salt: 1) without $(NH_4)_2HPO_4$; 2) 0.067 M $(NH_4)_2HPO_4$; 3) 0.2 M $(NH_4)_2HPO_4$; 4) 0.6 M $(NH_4)_2HPO_4$. White columns) without IP; cross hatched columns) addition of 0.05 M IP.

To explain the role of ATP in the synthesis of tryptophan from IP we ran special experiments in which we showed an activating action of ATP on the formation of tryptophan from IP (Table 1). ATP without addition of IP has no effect on the synthesis of tryptophan. The optimum concentration of ATP for activation, as Fig. 2 shows, is 3 mg per 1 ml of extract. At ATP concentrations of 1 mg/ml and 9 mg/ml the synthesis of tryptophan from IP was much lower.

Thus, ATP and glutamic acid, added separately, increase the formation of tryptophan from IP. When they are added simultaneously summation of the action of these compounds does not occur, as Table 1 shows. On the contrary, the stimulating action of glutamic acid alone was higher than the action of glutamic acid + ATP. The contradictory action of ATP in the presence and absence of glutamic acid, like the experiments with isoniazide, may indicate that the formation of tryptophan from IP in extracts without the addition of glutamic acid goes chiefly without participation of the transamination reaction.

Experiments with Plant Seedlings

The synthesis of tryptophan from IP which occurs in seedling extracts also occurs in the whole organs of plants, such as wheat ears [10] and also in green seedlings of wheat and peas. In all the experiments in which IP was forced into the plant seedlings there was an obvious synthesis of tryptophan. It was then shown that with increased time of infiltration of IP up to 10 hours there occurred an increase in tryptophan content. In the limits of this period there was a direct proportionality between time of infiltration and amount of tryptophan synthesized (Fig. 3).

We showed previously [1] that the synthesis of tryptophan from indole in etiolated seedlings goes more poorly than in green ones, and goes better in the tops of the green seedlings than in the whole seedling. We can assume that the intensity of tryptophan synthesis depends in some measure on the illumination of the plant. In this connection we carried out experiments with seedlings in the light and in the dark. As Table 3 (experiments 1 and 2) shows, in wheat and pea seedlings tryptophan synthesis goes faster in the light than in darkness.

To study the effect of ammonia on the synthesis of tryptophan from IP we carried out infiltration of IP neutralized to pH 8.0 by KOH or NH_4OH. In the experiments we carried out additional neutralization of the solutions to pH 8. As the results show (Table 3, experiments 3 and 4) ammonia strengthens the tryptophan synthesis.

In one of the experiments the ammonium ion was introduced in the form of $(NH_4)_2HPO_4$ in different concentrations. For stabilization of the pH during infiltration of the solution intended for infiltration it was prepared in a phosphate buffer (pH 8.0). From the results given in Fig. 4 we see that a high concentration of ammonium phosphate (0.2 M and 0.6 M) hinders synthesis of tryptophan from IP. At a concentration of 0.067 M ammonium phosphate stimulates this synthesis.

Thus, just as in wheat ears [10] at the corresponding conditions of pH and ammonium concentration the synthesis of tryptophan from IP is stimulated in seedlings. We can judge the participation of ammonia in this synthesis by comparing the behavior of amino acids in infiltration of K-IP and NH_4-IP. When K-IP is infiltrated there is considerable decrease of the amino acids compared to the control, which was infiltrated with water, with a very marked drop in serine and valine. With NH_4-IP infiltration the amino acid content is almost unchanged except for glutamic acid whose concentration markedly increases in connection with its synthesis. Thus, ammonia has a "sparing" action on amino acids.

Such behavior of the amino acids is found very obviously only when the IP salts are infiltrated in the dark. With infiltration by IP salts in the light, especially with long periods, there is a greater tendency to amino acid synthesis, at first of valine, leucine, and also threonine and γ-aminobutyric acid. In this case, formation of amino acids in seedlings when IP is infiltrated into them is evidently to be explained by deep decomposition of the latter.

SUMMARY

In extracts from seedlings and in whole seedlings of plants there is synthesis of tryptophan from β-3-indolepyruvic acid (IP). Glutamic acid stimulates tryptophan synthesis from IP in extracts of the plant. Isonicotinic acid hydrazide (isoniazide) prevents the stimulating action of glutamic acid. The synthesis of tryptophan from IP is strengthened also by the addition of ATP (without glutamic acid). Formation of tryptophan from IP and glutamic acid with addition of ATP is decreased.

Starting from our experimental results we can assume that the synthesis of tryptophan from IP in extracts of seedlings can occur in two ways: by transamination and by another path where ATP actually takes part.

When IP is introduced into the whole seedling by infiltration of the solution in the light and in the dark, synthesis of tryptophan from IP in the light goes much more intensively than in the dark. Under optimum conditions of pH and ammonia concentration the synthesis of tryptophan from IP is stimulated in the whole plant and has a "sparing" action on the amino acids.

LITERATURE CITED

[1] O. L. Polyanovskii and V. L. Kretovich, Doklady Akad. Nauk SSSR 112, 1086 (1957).*

[2] A. Ellinger and Z. Matsuoka, J. physiol. Chem. 109, 259 (1920).

[3] J. T. Holden, R. B. Wildman and E. E. Snell, J. Biol. Chem. 191, 559 (1951).

[4] A. Meister, H. A. Sober, S. V. Tice and F. E. Fraser, J. Biol. Chem. 197, 319 (1952).

[5] A. Meister, J. Biol. Chem. 206, 587 (1954).

[6] W. B. Jakoby and D. M. Bonner, J. Biol. Chem. 221, 689 (1956).

[7] S. Sakurai, Biochem. 44, 47 (1957).

[8] J. A. Bentley, K. R. Farrar, S. Housley, G. F. Smith and W. C. Taylor, Biochem. J. 64, 44 (1956).

[9] V. L. Kretovich and Zh. V. Uspenskaya, Biokhimiya 23, 243,(1958); 24, 116 (1959).

[10] V. L. Kretovich and O. L. Polyanovskii, Izv. Akad. Nauk SSSR Ser. Biol. No. 3, 428 (1959).

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NITROGEN EXTRACTIVE SUBSTANCES OF FISH MUSCLE*

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The content of nitrogen extractive substances in the muscular tissue of various forms of fish is very variable and depends on the species to which they belong, their habitat, living conditions, and many other factors. It is known from the data in the literature that fractions of the nitrogen extractives consist of 9 to 18% total nitrogen in the muscles of bony fish and from 33 to 38% in the muscles of cartilagenous fish. The characteristic of cartilagenous fish is the very high content of urea, trimethylamine oxide, and betaine [1-4]. In the composition of the muscular tissue of many kinds of cartilagenous fish are found the dipeptides carnosine or anserine. In the muscular tissue of all the studied species of fish are found creatine, creatinine, and creatine phosphate [4]. We have not found data in the literature on the content of arginine phosphate in fish. There are reports of the presence of large amounts of histidine in tuna muscle [2, 3]. In the work of Shewan there is a comparative investigation of amino acid composition of muscle of cod and flat fishes [2]. It was established that for skates the presence of betaine and sarcosine is characteristic, and for the muscles of cod the presence of anserine and methylhistidine is characteristic. Also, he succeded in isolating from cold muscles a considerable amount of taurine [5].

For a study of the composition of nitrogen extractives of the muscular tissues during the formation of the organism in the processes of embryonic and postnatal development of different animals (rabbits, pigs, chickens, ducks, rooks) there has been found a gradual appearance in the muscle at first of carnosine, and then in a later stage, of anserine. Before the appearance of carnosine a considerable quantity of the amino acids are observed which occur in the dipeptides, β-alanine or histidine [6-9]. Thus, with the development of the organism and the formation of muscle function, new, more complex compounds are synthesized.

The purpose of the present work is to carry out a quantitative determination of the nitrogen extractives: dipeptides anserine and carnosine, and also glutamic and amino acids in the muscles of various fish and to attempt to establish whether there is any regularity in the development of these compounds depending on the functional characteristics of the muscle connected with the type of life and the species of fish.

METHODS

As objects of investigation we used the following examples of fish classes. For the cartilagenous fish – skate (Raha clavata), of the suborder Elasmobranchii. For the bony fish – ganoids: sturgeon (Huso huso) and (Acipenser sturio); for the Isospondyli – anchovy (Engraulis encrasicholus), salmon (Salmon salar), and smelt (Osmerus eperlanus); for the Belonidae – garfish (Belone belone); for the Percoidea several different suborders were studied: for the Scombridae the bonito (Sarda sarda), for the Mullidea, the red mullet (Mullus barbatus), for the Trachinidae, the greater weever (Trachinus draco), for the Blennidae, the eelpout (Zoarces viviparus), for the Cottidae, the sculpin (Myoxocephalus scorpius). In the order of the Gadidae we studied the muscles of the cod (Gadus morhua maria-albi), baddock (Gadus nurlangus euxinus), navaga (Eleginus navaga), and polar cod Boreogadus saida); of the flatfishes, the sole (Solea solea).

The experimental material was collected at the Sevastopol Biological Station of the Academy of Sciences, USSR, and the White Sea station of Moscow State University. The skate, anchovy, garfish, bonito, sturgeons, mullet, weever, haddock, salmon and sole were taken in the summers of 1956 and 1957 in the Black Sea, and the smelt, cod, navaga, polar cod, eelpout, and sculpin in the White Sea in the winter of 1958.

*Fifth course student L. Mokhortova helped in this work.

TABLE 1

Content of Nitrogen Extractives in the Muscles of Skates, Garfish, Anchovy, and Bonito. In mg % nitrogen; averages of determinations from 5 or more subjects

Name of substance*	Skate	Bonito	Anchovy	Garfish
Creatine + creatinine	127,0	126,0	133,0	144,0
β-Alanine	27,0	—	—	—
Histidine	Traces	437	223	225
Sarcosine	14,9	—	—	—
α-Alanine	5,3	2,4	12,5	5,4
Glutamic acid	1,6	1,8	2,6	1,7
Threonine	—	1,3	2,9	1,3
Glycine	3,3	2,7	3,1	3,1
Serine	6,7	1,0	2,7	1,8
Aspartic acid	2,0	Not determined	2,5	0,8
Glutamine	3,1	3,2	7,8	6,8
Arginine	8,0	Not determined	14,8	6,1
Lysine	4,2	Not determined	Not determined	Not determined
Tyrosine	—	Trace	Not determined	1,8
Valine	0,8	Not determined	6,2	3,9
Phenylalanine	2,7	—	—	—
Leucine + isoleucine	3,7	Not determined	4,7	1,3
Amino nitrogen	1162	661	651	534
Dry residue of muscle, %	18,5	26,4	21,5	21,7

*Carnosine and anserine were not found in the muscles of these fish.

After decapitation of the fish we took the back and tail muscles, ground them coarsely, and extracted them with absolute alcohol. The resulting muscle samples were kept in a refrigerator until further treatment which took place after two to four months. The muscle tissues were then removed from the alcohol and carefully ground with twice the volume of a 5% solution of TCA, then combined with the alcohol extract and centrifuged. The protein precipitate was washed several times more with a weak TCA solution. The supernatant liquid after each centrifuging was combined with the previous ones, and the resulting mixture was evaporated to such a volume that the concentration of the solution corresponded to a content of 1 g of tissue in 1 ml of extract. A part of this solution, from which the TCA had first been removed by shaking with ether, was used for the determination of amino acids by paper chromatography. The ether by which the TCA was removed from the muscle extracts was saturated with water. In another part of the extract the dipeptides were precipitated with a solution of mercuric sulfate and alcohol, which also precipitated histidine and a number of other amino acids. After the necessary treatment of the precipitate of mercury salts of these compounds, we obtained a solution in which we determined carnosine, anserine and histidine.

The quantitative determination of amino acids was carried out by eluting the spots cut from the chromatogram by Bode's modified method. The paper for the chromatograms was first washed with a 0.15% solution of hydroxyquinoline in a butanol-acetic acid mixture. After washing in a butanol-acetic acid mixture which did not contain hydroxyquinoline, we dried the paper and washed it with water. For chromatography we used a mixture-butanol-acetic acid-water in different ratios: 4:1;1; 5; 1:1.3; 4:1:5. The solvent was passed five times, each time first drying the chromatogram. The chromatogram was developed with an 0.5% ninhydrin solution at 70° for 20 min. The spots were cut out and each spot was eluted in 4 ml of an 80% solution of methyl alcohol to which was added a saturated solution of $Cu(NO_3)_2$ (0.2 ml $Cu(NO_3)_2$ in 500 ml). Amino acids were fully eluted in three to four hours. When the eluate was kept in the dark the color did not change for a long time. Colorimetric determination was carried out on a microphotocolorimeter at 500 mμ. A standard amino acid solution was run in parallel with the experimental sample on each chromatogram.

The determination of histidine and the dipeptides carnosine and anserine was carried out after their separation on a chromatogram which was run in phenol saturated with water in an atmosphere which contained HCl vapors. Anserine was determined after eluting the spot cut from a chromatogram developed with ninhydrin.

TABLE 2

Content of Nitrogen Extractives and Amino Nitrogen in the Muscles of Cartilagenous Fish and Isospondyli. In mg % nitrogen; averages from determinations on 2-3 subjects

Name of compound	Acipenser*		Huso	Salmon	Smelt
	A	B			
Creatine + creatinine	195	182	186	55,9	115
Carnosine	60,4	34,8	33,6	—	—
Anserine	--	—	—	227	29,0
β-Alanine	17,3	5,5	31,2	—	—
Histidine	Trace	Trace	4,0	20,7	2,8
α-Alanine	3,5	4,8	6,7	2,5	6,6
Glutamic acid	0,5	1,6	1,4	1,4	1,4
Threonine	0,6	1,1	1,5	—	—
Glycine	4,1	4,7	9,1	1,9	32,7
Serine	1,3	2,5	Not determined	0,8	1,2
Aspartic acid	0,3	0,5	Not determined	1,2	Not determined
Glutamine	1,0	11,5	0,3	1,8	5,2
Arginine	1,2	Not determined	9,3	—	—
Lysine	Not determined	Not determined	Not determined	Not determined	3,4
Valine	—	—	—	1,1	Not determined
Tyrosine	—	—	—	1,3	0.8
Leucine + isoleucine	1,1	2,2	Not determined	Not determined	Not determined
Amino nitrogen	383	351	543	466	368
Dry weight of muscle%	20,0	17,8	—	24,5	—

*Acipenser of group A had a length of 0.9 m; of group B, 0.4 m.

Determination of the sum of carnosine and histidine was carried out colorimetrically from a trichloroacetic extract using the diazo reaction, and also carnosine and histidine were determined separately after their separation on the chromatogram. The chromatograms were developed by the diazo reaction, and the spots were eluted with 1% soda solution. For the identification of some amino acids two phased chromatograms were used. As solvents we used butanol-acetic acid and also phenol saturated with water. For the separation of aspartic acid, serine, glycine, glutamic acid, and threonine we used phenol saturated with a buffer solution, pH 12. Leucine and isoleucine separated poorly on the chromatogram and so the sum of these amino acids was determined. Determination of the amino nitrogen was carried out after combustion of the trichloroacetic acid extract of muscle by determining ammonia by the Conway method. The sun of creatine and creatinine was determined colorimetrically by the Jaffe reaction after hydrolysis of the trichloroacetic acid extract of muscle with 1 N HCl. For some of the Black Sea fish we determined the dry residue of muscle tissue.

In carrying out the quantitative determinations the results were calculated on wet weight of tissue.

RESULTS

The fish which we studied for content of nitrogen extractives could be divided into several groups. The muscle tissues of each group were distinguished by the characteristic composition of amino acids and dipeptides both in qualitative and quantitative respects. Among the above mentioned fish there were also species which did not contain the dipeptides carnosine and anserine, characteristic of the muscle tissues of vertebrates. However, in the muscles of these fish there were a large number of amino acids which make up these dipeptides—β-alanine (skate) or histidine (anchovy, garfish, bonito). On the other hand, there were fish whose muscles contained carnosine or anserine and one of the amino acids which compose them. Thus, for example, in the muscle of one of the sturgeons there were 290 mg % of carnosine and 99.7 mg % of β-alanine, and in the muscles

TABLE 3

Content of Nitrogen Extractives in Muscles of Gadidae. In mg% nitrogen; averages from determinations on 3 subjects

Name of compound	Cod	Navaga	Polar cod	Haddock
Creatine + creatinine	116,0	36,4	79,9	127,0
Anserine	13,8	16,1	10,3	9,2
β-Alanine	34,5	28,5	7,7	3,4
α-Alanine	11,0	10,4	7,1	4,5
Glutamic acid	2,8	1,4	1,6	2,4
Threonine	1,4	1,2	0,4	3,5
Glycine	15,5	21,0	12,5	2,6
Serine*	}	0,6	Not determined	
Aspartic acid	} 2,2	Not determined	3,0	} 1,9
Glutamine	14,0	3,8	28,8	8,3
Arginine	Not determined	Not determined	Not determined	7,6
Ornithine	Not determined	Not determined	Not determined	4,7
Phenylalanine	Not determined	0,7	0,8	0,5
Leucine + isoleucine	3,4	Not determined	2,1	2,6
Amino nitrogen	431	329	454	431

*Serine and aspartic acid in the muscles of cod and haddock were poorly separated on the chromatogram; therefore they were determined together.

of one of the salmon we found 1127 mg % anserine and 84 mg % histidine. In addition there are also species in which neither carnosine nor anserine occur, and which also have none of the amino acids which compose them or only contain these in small amounts. However, the muscles of these fish are rich in glutamine and also in such amino acids as glycine and α-alanine.

The first group of fish includes the cartilagenous fish of the flat type – the spiny skate which lives on the bottom. In the composition of skate muscle, besides β-alanine, there is much sarcosine; other amino acids are present in much smaller amount. In Table 1 we give average data obtained in the study of muscles of five skates. The content of amino nitrogen in skate muscles is very high, greatly surpassing the sum of the nitrogen of all the nitrogenous substances which we determined. However, we did not analyze for such compounds as urea, trimethylamine oxide, and betaine which are known to be present in the muscles of cartilagenous fish, and especially in skate muscles, in very large amounts and which evidently make up the greater part of the amino nitrogen. In the determination of the dry weight of the muscle tissue we obtained similar close values for the muscles of each skate, an average of 18.5%.

In the second group of fish with an especially rich content of histidine in the muscles were the anchovies, garfish, and bonito, which belong to suborders of pelagic species. Garfish and bonito are predators, eating small fish. In Table 1 we give average results obtained in the study of muscles taken from 16 anchovies, five bonito, and six garfish. The qualitative makeup of amino acids in all these groups is the same, with only some quantitative variation. The content of the sum of creatine and creatinine is almost the same in the muscles of all three species of fish. In the bonito muscles there is always the largest amount of histidine (which in the muscles of some subjects among the bonito made up 3% of the wet weight of the tissues), but other amino acids and glutamine were less in the bonito than in the muscles of anchovies and garfish. The total nitrogen of amino acids, glutamine, and creatine plus creatinine made up most of the nitrogen extractives, and almost half of it in the muscles of anchovies and garfish and more than half of it in the bonito was in the form of histidine.

In the fish of the third group, belonging to the cartilagenous ganoids, we studied the muscles of the two forms of sturgeon. In Table 2 we give average values for the content of these substances in this group. We note the very large amount of creatine and creatinine in sturgeons. Table 2 shows that the carnosine content in the muscles of Acipenser (type A) is greater than in Huso, but in Huso the content of the other amino acids is higher: β-alanine, α-alanine, glutamic acid, etc.

TABLE 4

Content of Nitrogen Extractives in Muscles of the Percoids and the Flatfish. In mg % nitrogen; average values for determinations from 5-8 subjects

Name of compound	Red mullet	Weever	Sculpin	Eelpout	Sole
Creatine + creatine	Not determined	Not determined	Not determined	130,0	28,7
α-Alanine	8,4	4,4	10,0	19,1	4,3
Glutamic acid	2,3	3,9	3,5	2,2	1,6
Threonine	1,9	Traces	1,8	Not determined	1,3
Glycine	17,4	4,6	14,5	22,1	5,2
Aspartic acid	2,2	1,3	5,6	Traces	4,1
Glutamine	37,6	25,2	36,6	25,2	16,9
Arginine	Not determined	Not determined	8,5	9,2	4,8
Lysine	Not determined	Not determined	Not determined	6,2	3,4
Histidine	5,4	0	Traces	0	2,7
Tyrosine	3,6	1,5	2,3	Not determined	2,6
Phenylalanine	1,6	Not determined	3,3	Not determined	Not determined
Leucine + isoleucine	0,8	1,8	1,0	1,	Not determined
Amino nitrogen	349	286	275	338	310

Comparison of the results obtained in the study of the extractives of the muscles of sturgeon of different ages shows that the content of amino acids and glutamine decreases as the fish grows, and the amount of carnosine increases. These results agree with those which we found in other vertebrates (ducks, rabbits). An exception is β-alanine, the amount of which increases with the growth of the fish.

In the fourth group of fish were the salmon and smelt which are examples of the isospondyli. The characteristic perculiarity of these fish, as already mentioned, is the presence in their muscles of anserine and histidine. In smelt muscles the content of anserine and histidine is much lower than in salmon, but the greater amount of glycine, 176 mg %, should be noted, and also the larger amounts of glutamine and α-alanine than in salmon. The high content of these substances, as a rule, has been found by us in fish from the White Sea, or in the muscles of fish in which we did not find much of the dipeptides or the amino acids which compose them. In the smelt, an example of a White Sea fish, the quantity of dipeptides in the muscles was small. The quantitative results which we obtained for the muscles of salmon and smelt are given in Table 2.

The following group is made up of Gadidae, of which the cod, navaga and polar cod were taken in the White Sea, and the haddock in the Black Sea. The muscles of these fish (Table 3) contained anserine and β-alanine. An especially large amount of β-alanine was found in the muscles of the cod (220 mg %) and the navaga (182 mg %). The qualitative composition of the other amino acids in the muscles of these fish was the same, but there was some difference in quantitative composition (Table 3). Thus, in cod, navaga, and polar cod there was a larger amount of α-alanine and glycine. Also, in the muscles of the cod and especially of the polar cod there was a great amount of glutamine (152 mg %). The increased content of these substances, as in smelt muscles, was evidently determined by the living conditions of the fish, since in the muscles of the haddock, taken in the Black Sea, this was not found. As to the group of amino acids, they differed little from the amino acids found in the preceding groups.

Besides the compounds shown in Table 3, there were spots on the chromatogram which we did not identify. As the literature data show, in the muscles of cod and haddock there are taurine, glutathione, cysteine, and methylhistidine. It is possible that the spots which we did not identify belonged to these compounds.

In the last group of fishes occur examples of several families of the percoid fishes: mullets, weevers, sculpins and eelpouts, and of the flatfish, sole. Mullets, weevers, and sole live in the Black Sea, eelpouts and

sculpins in the White Sea. We note the fact that in the muscles of all these fish, independent of where they live, we find a large amount of glutamine, and in the mullet, sculpin and eelpout there are also much glycine and α-alanine. Information on the amino acid composition of the muscles is given in Table 4. Creatine and creatinine (Table 4) were determined only in the eelpout and sole; in the sole the content of these substances is very small, nearly five times less than in the eelpout. We should note the presence of individual variations in determinations of amino acids in the muscles of this group of fish and also in the fish of the preceding groups.

DISCUSSION OF RESULTS

It is known that carnosine and anserine are distributed in the muscles of vertebrates and are not found in the invertebrates. The presence of these substances in the muscles of some fish and their absence in others gives reason to suppose the existence of group differences between fish with respect to the composition of the extractives of nitrogenous substances in muscle tissues. The appearance of the dipeptides in ontogenesis of animals (rabbits, pigs, chickens, ducks, rooks) is connected with the processes of muscle formation. In the early stages of embryonic development of these animals β-alanine or histidine are observed in the muscles. Among the fish studied we find those which contain a large amount of β-alanine or histidine in the muscles, and also those in which there is also carnosine or anserine. We may believe that the presence of these substances is connected with the characteristic peculiarities of development of the skeletal muscles of various fish.

The skate, whose muscles contain β-alanine, belongs to the cartilagenous fish of very ancient origin, lives on the bottom, and is a relatively little mobile form of life. On the other hand, fish which contain the largest amount of histidine, bony fish; anchovies, garfish, bonito, belong to the pelagic group, are very mobile, carry out long migrations, but are also ancient enough in their origin. In further evolution, muscles can follow different paths. In one case increasing complexity can lead to formation of new substances such as carnosine or anserine, but keeping some quantity of β-alanine which was characteristic of the muscles of their ancestors. Thus, the ganoid fish (sturgeons) whose muscles contain carnosine and considerable amounts of β-alanine are evidently closely related in their origin to the cartilagenous fish. We can consider that a relationship with the ganoid fish is indicated for the Gadidae whose muscles, as distinct from those of the sturgeons, contain not carnosine but anserine, but, like the sturgeons and cartilagenous fish, also contain β-alanine.

In a different case, muscle evolution can occur in such a way that the muscles form carnosine or anserine, but retain histidine. As an example we have the Salmonidae: their muscles form anserine but retain histidine.

As to the Percoids and flatfish whose muscles do not contain the above compounds, it may be that the evolution of the muscles of these fish proceded differently so that a large amount of glutamine, glycine and α-alanine were formed. An explanation of the question of why the evolution of muscles led to the accumulation of such substances requires further investigation. We can mention the data on the composition of muscles in the embryonic period in development of ducks, when along with β-alanine there is a particularly high content of glutamine, glycine, α-alanine, and some other amino acids [9]. A considerable amount of glycine and α-alanine are also found in the muscles of a number of invertebrates caught at the White Sea Biological Station.* The representatives of the groups of fish under discussion (red mullet, weever, sculpin, eelpout, sole) are coastal or bottom forms of life which are characterized by bodies extended or flattened on the sides. Some of them live on the bottom and often dig into the mud with only their eyes at the surface. All these fish are benthonnic. The presence of the dipeptides or a large amount of the amino acids which compose them is evidently characteristic for fish with a more developed musculature. We should note the fact that for fish which live in the White Sea there is a high content of glycine and α-alanine in the muscles. However, it is necessary to remark that our results were obtained in studying fish caught at different seasons of the year: White Sea fish in the winter and Black Sea fish in the summer. The chemical composition of muscle tissue can change depending on conditions of the surrounding medium as well as on the biological peculiarities of the organism. Therefore, it is possible that the accumulation of glycine and α-alanine is related to a seasonal peculiarity of the White Sea fish.

SUMMARY

For cartilagenous fish a characteristic peculiarity of muscle tissue is the presence of a considerable amount of β alanine and sarcosine. In the muscle tissue of the pelagic group of bony fish, bonito, anchovy, garfish,

*The data are taken from the diploma work of Fifth Course Student M. M. Shemyakin (1959).

there is a large amount of histidine (from 0.8% to 3% of the wet weight of tissue). In the muscles of the Salmonidae (salmon, smelt) there are anserine and histidine. In the sturgeons, carnosine and β-alanine are characteristically present. The muscles of representatives of the Gadidae (cod, haddock, navaga, polar cod) contain anserine and β-alanine.

Representatives of the percoid fish (red mullet, weever, sculpin, eelpout) and the flatfish, sole, do not contain in their muscles either the dipeptides or the amino acids which compose them, but are characterized by the presence of large amounts of glutamine.

Fish caught in the White Sea, independent of which group they belong to, contain a large amount of glycine and α-alanine in their muscles. The other amino acids in the muscles of the different fish studied are the same and consist of glutamic acid, threonine, serine, aspartic acid, leucine and isoleucine, phenylalanine, arginine, valine, tyrosine, and lysine, present in different amounts which do not surpass 10 mg % of nitrogen for each amino acid, however.

LITERATURE CITED

[1] D. Shewan, in the book: Biochemistry of Fish (IL, Moscow, 1953) [Russian translation] p. 39.

[2] J. M. Shewan, J. Sci. Food. and Agric. 3, 394 (1952); 4, 565, (1953); 6, 99 (1955).

[3] L A. Tarr, Annual Rev. Biochem. 27, 223 (1958).

[4] E. Bolduin, in the book: Biochemistry of Fish (IL, Moscow, 1953) p. 7 [Russian translation].

[5] N. R. Jones, J. Sci. Food and Agric. 6, 3 (1955).

[6] N. A. Yudaev, Doklady Akad. Nauk SSSR 70, 279 (1950); 71, 717 (1950); Uspekhi Sovrem. Biol. 30, 176 (1950).

[7] S. E. Severin and V. M. Fedorova, Doklady Akad. Nauk SSSR 82, 443 (1952).

[8] P. L. Vul'fson and S. E. Severin, Ukr. Biokhim. Zh. 3, 295 (1955).

[9] P. L. Vul'fson, Biokhimiya 23, 300 (1958).

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THE AGE FACTOR IN THE EFFECT OF THE PITUITARY SOMATOTROPHIC HORMONE ON NUCLEIC ACID METABOLISM

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The effect of the hypophyseal somatotrophic hormone (STH) on nucleic acid metabolism has been successfully studied in recent years. Thus, Stefano et al. [1] showed that the fall in cellular liver RNA, observed after hypophysectomy, could be reversed by treatment with STH. Neither hypophysectomy nor STH administration were found to have any effect on the DNA levels. Cater et al. [2], and also Greenberg and co-workers [3] have established that STH administration increased the rate of P^{32} incorporation into rat liver RNA. Similar results were also reported by Cornatzer and co-workers [4].

On the basis of the modern concepts of protein biosynthesis, the above-mentioned data may be considered as an important argument in favor of the theory [5-7] that STH exerts a stimulating effect on the anabolic phase of protein metabolism. The available experimental results also afford a better understanding of the complex biochemical mechanism of the somatotrophic function of the pituitary. A study of the scarcely investigated region of the interrelation between STH and nucleic acid metabolism, at various stages of ontogenesis, would be of interest in both the fields of developmental physiology and biochemistry.

Taking into account the various theories on the change in receptive sensitivity of the peripheral tissues to central regulating stimulants (especially endocrine [8]), on the age-specificity of the "hormonal spectrum" [9], and also some available information about the ontogenetic development of the somatotrophic function of the anterior pituitary [10-12], it is possible to arrive at two operative factors in the determination of the decreased volume and intensity of protein biosynthesis in the aged as influenced by STH: the age-dependent decrease in tissue susceptibility to STH or the decreased production of STH in the aged animal. It is not excluded that both factors are operative.

METHODS

Since it was envisaged that the true effect of the STH treatment may be missed in case of single doses, it was considered advisable to treat the animals with multiple doses of the hormone. It was also throught worthwhile to attempt an investigation into the dynamics of the effect of STH on the nucleic acid metabolism.

Crystalline pituitary somatotrophic hormone was prepared according to Wilhelmi et al. [13], who first employed for this procedure the excellent technique of ethanol fractionation of an anterior pituitary protein extract. Biological assay and standardization of all preparations was carried put as described by Evans and Li [14].

Growth hormone is currently considered to possess a certain amount of species specificity [15]. However, in the present work, STH prepared from ox pituitaries was suitable, according to data at hand [16], for use in experiments with albino rats.

Male albino rats were used in all experiments and were of four age groups: one-month old, 3 months, 12 months and 18-20 months old. The animals were kept in metabolic cages and were given the Abelin diet (bread and milk, 1:1 by weight) with a supplement of vitamins and an optimal amount of phosphate salts. STH was

TABLE 1

The Liver Content of Various Phosphorus Compounds in the Albino Rat, in Control Animals and in Experimental Animals 24 Hours after a Single Injection of STH. (P concentration as mg/g dry weight. Mean values)

Age, months	STH-injected	Control	Difference	significance	STH-injected	Control	Difference	Significance
	RNA				DNA			
1	3,348	3,295	0,053	0,50	0,831	0,914	—0,083	— 3,5
3	3,219	2,719	0,500	8,00	0,863	0,692	0,171	43,2
18	3,003	2,946	0,057	0,70	0,806	0,868	—0,062	— 1,42
	Lipid-P				Acid-soluble-P			
1	5,081	5,089	—0,009	—0,03	3,469	3,438	0,031	0,11
3	5,914	5,335	0,579	1,28	3,667	3,382	0,285	0,44
18	3,014	2,932	0,082	0,14	2,887	2,755	0,132	1,59

Note: In the variant-analysis the difference is considered unconditionally significant when significance values are 3.0 or higher.

TABLE 2

The Effect of STH on the Allantoin and Total Nitrogen Content of Urine from Rats of Different Ages. (Numbers denote mean values averaged from allantoin and total nitrogen content of urine collected on the 2nd, 4th, 6th and 8th day from the beginning of STH administration, (% of normal values). Each set of values used for each age group represents a mean of 6-10 experiments)

Age, months	Urinary allantoin	Urinary nitrogen
1	92,8	92,4
3	79,4	85,0
12	79,6	80,9
20	75,9	81,1

administered as an alkaline (pH 8.5-9.0) aqueous solution subcutaneously, in doses of 0.5 mg of the hormone per 250 g of body weight. Control animals received slightly alkaline water in the same amounts and at the same intervals as experimental animals. The animals were divided into two groups, each containing both experimental and control sub-groups.

Group I: animals received the STH in one single injection; Group II: the animals received the STH over a period of seven days. However, in order to prevent the adaptation of the animals in this group to STH, the hormone was administered in five doses over the seven-day period, no treatment being given on the 4th and 6th day.

Urine was collected under toluene from the animals of the second group over 24-hour periods on the 2d, 4th, 6th and 8th day after injection. The animals of all groups were killed 24 hours after the last injection, the livers were removed, washed with cold normal saline and used for analysis.

The following estimations were carried out on the urine: total nitrogen by a micro-Kjeldahl procedure, allantoin according to Larson [17], uric acid according to Fearon [18]. Liver analysis involved the estimation of the main phosphorus fractions — nucleic acid phosphorus (RNA and DNA separately), acid-soluble phosphorus and lipid phosphorus — by a somewhat modified procedure of Schmidt and Thannhauser [19]. Total and residual nitrogen in the liver was determined also by a micro-Kjeldahl procedure. Dry weight of liver was taken to be that remaining after drying the fresh tissue to constant weight at 105°.

The rats were selected for the experiment so as to ensure that representatives of all the age-groups investigated were present in all seasons; this allowed to partially account for some seasonal variations in some of the investigated denominators. The animals were weighed periodically. Since the animals were introduced into the experiment in pairs (experimental and control), and since each member of the pair was held in identical conditions, it was possible to carry out statistical analysis of variants in the results by calculating the error of the difference of the means of two associated series [20].

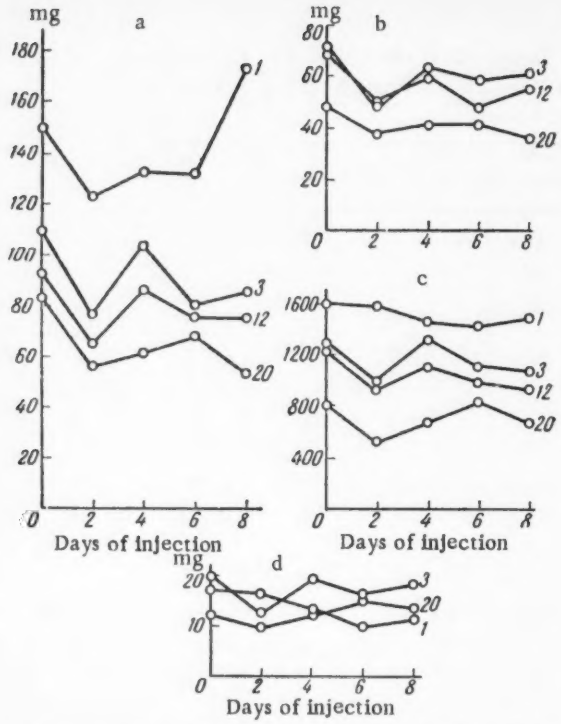

Fig. 1. The effect of STH treatment on the concentrations of allantoin, total nitrogen and uric acid in the urine of rats of various ages. Concentrations were expressed as mg excreted in the urine in 24 hours per kg body weight: a) allantoin content in the winter; b) same, in the summer; c) total nitrogen; d) uric acid content, mean of both seasons. Each point on the graph represents a mean of 6-10 experiments. Numbers at the end of curves represent the age of animals in months.

RESULTS AND DISCUSSION

Single injection of STH. The effect of a single injection of STH on the concentration of various phosphate fractions in the liver, 24 h after the injection, is shown in Table 1 (mean values of 7-10 experiments). It will be seen that there was a definitely significant increase in the RNA-P content in the liver on treatment of 3-month old rats with STH; such change was not evident in either the one month-old or 18-month old groups. It would appear that in three-month old animals the synthetic processes were most receptive to a single injection of STH. In the same age-group there was also a significant increase in the DNA-P following STH administration; there was also a slight increase in the DNA-P in the STH-treated one-month old animals, but no effect was detected in the 18-month old group. In none of the groups was there any increase in the content of lipid-P or acid-soluble-P.

One single injection of STH caused in animals of all ages a considerable retention of allantoin, which in the rat is the main product of nucleic acid breakdown. This effect on retention per se allows some conclusion about the mode of action of STH on nucleic acid metabolism: it seems to exert a "sparing" effect through inhibiting nucleic acid breakdown.

The values for urinary allantoin in the 24-hour urines of normal animals and of treated animals, on the 2d, 4th, 6th and 8th day after the beginning of treatment, are shown in Fig. 1a and b, for the winter and summer period, respectively (there were large seasonal variations in the levels of excreted urinary allantoin).

The most pronounced fall in the excretion of allantoin was observed in the 3-month old animals after treatment with STH. Thus, in this age group, on the second day of the experiment, allantoin excretion in the urine fell by 32.2% from the normal level (taken as 100%) of untreated animals (mean values of winter and summer seasons). When this result is considered in the light of the information (Table 1) on the increase in liver RNA and DNA in three months-old rats after a single injection of STH, it seems quite probable that in young animals, as distinct from animals of other age groups, a single injection of STH inhibited the breakdown of nucleic acids.

Allantoin retention was least pronounced in one-month old rats. After the first STH injection (second day of experiment) the allantoin levels in the urine of those animals fell only by 18.5% from control values (winter period). This is quite understandable, considering the high intensity of the synthetic processes in the organisms of growing, sexually immature animals. This insignificant effect on the retention of allantoin is in agreement, to some extent, with the failure of the treatment to affect RNA concentration and the very slight fall in DNA (Table 1).

A considerable fall in urinary allantoin levels after the first STH injection (second day of experiment) was also observed in 20-months old rats. Quantitatively, this change (mean summer-winter value – 27.9% lower than control) was somewhat less pronounced than in three-months old animals, which may be due to a decreased susceptibility of the peripheral tissues to STH, conditioned by age. When these differences are compared with

TABLE 3

The Concentration of the Chief Phosphorus-Containing Compounds in the Livers of Control and Treated Albino Rats on the Eighth Day of Treatment with STH (All values as mgP/g dry weight. Mean values)

Age, months	STH-treated	Controls	Difference	Significance	STH-treated	Controls	Difference	Significance
	RNA				DNA			
1	3,180	3,220	—0,040	—0,61	0,937	0,936	0,001	0,03
3	2,489	2,254	0,235	4,32	0,711	0,646	0,063	2,65
12	2,592	2,344	0,248	5,80	0,792	0,809	—0,017	—0,15
20	2,606	2,149	0,457	9,20	0,709	0,676	0,033	1,11
	Lipid-P				Acid-soluble-P			
1	4,248	4,966	—0,718	—1,18	3,952	3,567	0,385	2,04
3	5,133	4,183	0,950	3,36	3,446	3,046	0,400	4,68
12	4,138	3,513	0,624	1,77	3,147	2,907	0,240	2,24
20	4,406	3,671	0,735	1,36	3,373	2,864	0,509	2,64

the data on the effect of STH on the liver nucleic acid content (certain changes were observed), the quantitative lack of correspondence of the two effects becomes obvious. While the animals of the 20-month age group differ from the 3-month olds only slightly in their STH-induced retention of the products of nucleic acid breakdown, (Fig. 1a and b), they lag badly behind the young animals in their ability to re-utilize these products in subsequent RNA synthesis in the liver (Table 1). Consequently, the decreased susceptibility of the tissues of old animals to STH is related to the well known involution of the rate and intensity of the metabolic processes in the aged. It is also established that the relative (to body weight) weight of the liver decreases with age.

Allantoin retention in 12-months old animals was on levels intermediate between those observed in the 3-months old and 20-months old animals. Two days after the beginning of the experiment the retention of urinary allantoin in these animals amounted to 28.7% of the amount normally excreted (mean value of summer and winter periods).

It will be seen from Fig. 1a and b, that, both in STH-treated and in control animals, allantoin concentrations were lower in the summer than in the winter (under the conditions of the present experiment, room temperature in the summer was 24-26°, in the winter — 16-18°). This phenomenon may gain some clarification from the experiments of Young and Cook [21], who found that urinary total nitrogen, urea, allantoin and creatine increased with decreasing ambient temperature, within reasonable limits. In the present work the changes in total nitrogen excretion in the urine of animals of all age groups receiving STH followed quite closely the changes in allantoin concentration (Fig. 1c).

The changes in urinary uric acid excretion following a single injection of STH followed, at least in the 3-months and 20-months old animals, the same tendency as the other nitrogenous urine constituents (Fig. 1d). It was also noted that the allantoin excretion in the control animals decreased with age, which was in agreement with the findings of Parina [22]. This decline in allantoin excretion appears to be an indication of the tapering off of the processes of nucleic acid rejuvenation and points to the decreased general rate of nucleic acid metabolism with the progress of ontogenetic development.

Multiple treatment with STH. Three-month old rats, which were most receptive to the first injection of STH, were also capable of rapid adaptation to the hormone on subsequent treatment: on the fourth day of the experimental period the urinary levels of the compounds under investigation returned to nearly normal values (Fig. 1). A subsequent period of "rest" from the hormone did not return the animals to their original level of sensitivity; on the eighth day the retention of all the urinary constituents investigated was lower than on the second day. Such a degree of adaptation indicates the apparently high functional value and flexibility of the central regulating systems in the young, sexually mature animals, who were able to cope with a massive dose of STH by developing a specific resistance reaction, that is, by passing into a state of "stress."

Unlike the three-months old animals, the 20-month olds were much less adaptable to subsequent STH

TABLE 4

Entire Liver Contents of the Chief Phosphorus-Containing Compounds in Normal and STH-Treated Rats on the Eighth Day of Treatment (In mgP per organ. Mean data)

Age, months	STH-injected	Controls	Difference	Significance	STH-injected	Controls	Difference	Significance
	RNA				DNA			
3	6,659	6,266	0,394	4,27	1,982	1,760	0,220	1,78
12	8,127	6,924	1,203	3,67	2,488	2,653	—0,166	—0,93
20	8,142	6,495	1,647	7,16	2,200	2,046	0,154	0,84
	Lipid-P				Acid-soluble-P			
3	13,614	11,564	2,050	2,71	9,252	8,438	0,814	2,28
12	12,952	12,065	0,887	3,40	9,884	9,356	0,528	2,95
20	13,619	11,193	2,426	2,28	10,492	8,751	1,741	3,07

injections. Thus, on the fourth day of the experiment the retention of allantoin was still significant, while on the eight day it actually exceeded the values of retention on the second day (Fig. 1a). This is possibly due to an entirely altered situation in the neurohumoral interrelationships resulting in a decreased capacity to develop resistance to introduced STH.

Thus, while the initial response of the aged animals to STH was not as high as that of the young ones, the duration of the response in the old animals was much longer, which resulted in actually lower values of the mean allantoin and total nitrogen excretion when the values of the four collections were averaged (Table 2).

The overall reaction to STH, in terms of total retention over the entire experimental period, was least in the one-month old rats (Table 2). The adaptation in the animals of this age group seems to be the highest of all groups: on the eighth day after the beginning of the experiment allantoin excretion actually exceeded normal values (Fig. 1a). The 12-months old rats occupy an intermediate position between the one-month old and 20-months old groups, both with respect to degree of allantoin retention, and adaptation to STH.

It would thus appear that in older animals the retention of nitrogen and retardation of nucleic acid breakdown elicited by STH treatment was no less in degree, and somewhat larger in its total effect, than in the younger animals. Furthermore, allantoin excretion on the eighth day of treatment decreased with increasing age of animals. This result was diametrically the opposite to that observed with a single STH injection (2d day of experiment). It was therefore of interest to investigate the content and concentration of nucleic acids in the old animals on the eighth day of experiment, that is, when repeated treatment with STH has brought about a remarkable retention of the products of nucleic acid breakdown.

The effect of multiple injections of STH on the concentration of the chief phosphorus-containing fractions in liver is shown in Table 3.

In the one-month old animals, on the eighth day of a course of STH injections, liver RNA levels were actually somewhat below initial values, which was in good accordance with the observed slight elevation above normal values in the urinary allantoin excretion (Fig. 1a). The observed increase in purine breakdown was thus reflected in a slight depletion of liver RNA.

In the animals of the other age groups the liver RNA levels rose above normal values after the prolonged STH treatment and, as expected from the data on allantoin retention at that stage, the STH-elicited elevation of liver RNA levels increased with age: in the 20-months old rats it was twice that observed in the 3-months old animals. This rather unusual phenomenon of increasing effect of multiple STH treatment on RNA content with increasing age is of considerable importance in the elucidation of the specificity of the hormone's effect on protein metabolism in the various phases of ontogenesis.

The multiple treatment with STH failed to cause any appreciable changes in DNA content in the animals of any age groups, and wherever small changes occurred variant analysis proved them to be statistically non-significant. Lipid-phosphorus content increased significantly after STH treatment only in the 3-months old group; no recognizable pattern of change could be observed in the other groups. The results obtained with the acid-soluble phosphorus were very similar to those of lipid-P.

TABLE 5

The Increase in Liver Mass, Total Liver RNA and RNA Levels per Unit Wet Weight of Liver Over the Entire Period of STH Treatment

Variable studied	Age, months		
	3	12	20
Increase in liver mass, g	0,377	0,546	1,300
Increase in total liver RNA, mg	0,496	1,377	1,693
Increase in liver RNA levels mg/g liver	0,053	0,125	0,163

TABLE 6

Total RNA-P Content of Liver per Gram Body Weight, Following Multiple STH Injections (In mg)

Age, months	STH-injected	Controls	Difference	Significance
3	0,241	0,238	0,003	0,204
12	0,243	0,197	0,046	1,78
20	0,253	0,173	0,080	5,06

Prolonged treatment with STH also stimulated liver growth, particularly in old animals. In view of this it was throught advisable to calculate the content of the chief phosphorus-containing fractions in the whole organ following the multiple STH treatment (Table 4).

The trend of changes in RNA and DNA content of the whole livers did not differ from the changes when calculated on the weight basis, whether in the values of arithmetical means or in the significance of the differences found, that is, multiple injections of STH raised the total content of liver RNA more markedly in older animals than in younger ones. DNA content of whole livers remained unaltered (the livers grew through increase in cell mass, but not in cell number). The changes in lipid-P and acid-soluble-P content, though significant, did not follow any definite pattern.

It is possible, using the known ratio of liver mass to body weight in normal animals, to calculate the liver weight of the treated animals, and hence the increase in liver mass, the increase in RNA per liver and the increase of RNA per unit of wet liver weight, over the total period of STH treatment (Table 5). This calculation again revealed the previously observed tendency: the effect of STH on the liver RNA-P content increases with the age of the animal.

The present results, indicating the failure of STH treatment after eight days to affect the liver RNA-P levels in three-months old rats, are in agreement with previously published results of Gray [23]. The latter author administered STH to rats (corresponding in weight to the three-months old animals of the present work) in nine daily doses (0,5 mg each), and reported RNA and DNA levels in liver unchanged at the end of the experimental period.

In order to gain some understanding of the paradoxical observation on the maximal stimulation of RNA levels in the livers of old animals by a seven-day treatment with STH, and in order to avoid a possible source of error inherent in some discrepancies in the initial weights of control and experimental animals, the RNA-P contents were calculated on the basis of body weight units (Table 6).

The changes in this case were found to be in the same direction as those previously calculated, and bore the same age-relationship (cf. Table 3). However, on the new calculation basis the changes in the 3-months old and the 12-months old animals were found to be nonsignificant, and only the increase in total liver RNA in the 20-months old animals was shown to be significant on the basis of the unit of total body weight.

In view of the fact that the results presented earlier (Table 4), where a significant increase in total organ RNA following prolonged STH treatment occurred with all ages of animals, stand in direct contradiction with those presented in Table 6, the latter results deserve further examination.

It will be seen from Fig. 2 that the lack of significant change in liver RNA content on a body weight unit basis in the three-months old rats was due to the relatively high intensity of body growth compared with the liver growth after treatment with STH, while in the older rats the opposite held, in the 20-months old animals

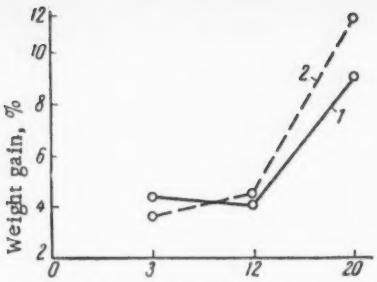

Fig. 2. The dynamics of total body and liver weight gain in rats of various ages given multiple injections of STH: 1) gain in total body weight; 2) gain in liver weight.

the stimulating effect of STH on liver growth (splanchnomegaly) exceeding that on the body growth and, aided by the already significantly higher relative RNA-P content, bringing about a significant increase in total liver RNA as calculated on a unit of body weight basis.

The above results allow the conclusion that the treatment with STH retarded the catabolism and enhanced the anabolism of nucleic acids, the effect being dependent on at least two factors: the age of the animals and the duration of treatment. In order to obtain a certain intensity of response the duration of treatment must increase with the age of the treated animals. In other words, the latent period of STH effect on protein synthesis increases with age.

Towards the end of the experimental period, old animals showed RNA levels and total contents in liver similar to those found in young animals (Tables 3 and 4), the per cent change actually exceeding that observed in the three-months old rats on the second day of treatment, which was the maximal value for this age group (Table 1); the sum total of retention of some of the urinary constituents studied, over the entire experimental period, was higher in old rats than in three-months old ones (Table 2). All these facts indicate that the aged organism has sufficient potentialities for RNA synthesis, and that the increased latent period in the aged animal's response to STH is not, apparently, due to a lower peripheral susceptibility to the hormone, but has some other reasons.

Currently available data on the STH levels in the blood and hypophysis of man and animals are incomplete and contradictory. Thus, Gershberg [10], on the basis of results obtained from human hypophysis implantation into animals, claims that STH synthesis in the hypophysis is not retarded in the old, while Baker [11] claims to have shown a decrease in the content of this hormone in the hog hypophysis with aging. Segaloff et al. [12], using the tibia test for STH assays in human blood give the impression that in humans blood STH levels fell somewhat towards the age of 50; however, in view of the rather high degree of individual variation and the relatively small sampling of material, the latter results must be treated with a certain amount of caution.

The increased effectiveness of administered STH with age suggests the hypothesis whereby the production of the hormone in the pituitary decreases with age, since the reactivity of the organism to the hormone largely depends on the degree of its saturation with STH. One may therefore hazard the assumption, with a certain degree of probability, that it was the low degree of saturation with STH of the tissues of the 20-months old rats that elicited the pronounced effects upon the multiple treatment with the hormone.

The observed age-dependent changes in adaptability of the animals to STH, which bear relation to the disturbance of integrity of the regulating systems of the organism, were also responsible for the observed age-specificity of the STH effect: the young, sexually mature animal, although more sensitive to a single STH injection, was able to show a considerable degree of adaptation, towards the end of the experimental period, to the effects of STH; the older animal, in contrast, has lost this ability, and consequently showed a larger overall response resulting in significant changes of nucleic acid metabolism after repeated treatment with STH.

The lack of any response to STH treatment in the one-month old rats is easily explained by the high degree of saturation of the tissues in these animals with endogenous STH and the very pronounced capacity for adaptation to administered exogenous hormone.

The general conclusion from the results obtained and from the considerations expressed above, is that in the process of normal growth the progressive loss, with aging, of the rate of growth and protein synthesis is not merely due to a loss in sensitivity to STH by the peripheral tissues, but that it results rather from a change in the entire situation of endocrine interrelationships in the process of ontogenesis.

SUMMARY

The communication describes the effect of single and multiple injection of STH on the urinary excretion of nitrogen and of products of nucleic acid breakdown, and on the concentration of several phosphorus-containing fractions in the livers of albino rats of different ages.

STH treatment was found to prompt the retention of allantoin, uric acid and of total urinary nitrogen. The total retention of those compounds over the entire period of repeated injections increased somewhat with the age of the animals. The anabolic effect of STH thus depended on the duration of treatment as well as on the age of the animal. The ability of the animals to adapt to repeated injections of STH decreased with increasing age.

DNA levels in animals of all ages remained unaltered after STH treatment. The changes in the concentrations in liver of acid-soluble-P and lipid-P on treatment with STH did not follow any distinct pattern.

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LITERATURE CITED

[1] H. S. Stefano, H. F. Diermeier and J. Tepperman, Endocrinology 57, 158 (1955).

[2] D. B. Cater, B. E. Holmes and L. K. Mee, Biochem. J. 66, 482 (1957).

[3] A. L. Greenbaum, C. N. Graymore and T. F. Slater, J. Endocrinol. 15, 235 (1957).

[4] W. E. Cornatzer, D. G. Gallo and J. P. Davison, Proc. Soc. Exptl. Biol. and Med. 84, 103 (1953).

[5] R. Weil, Arch. Internat. Med. 95, 739 (1955).

[6] E. Klein, Dtsch. med. Wochenschr. 82, 484 (1957).

[7] Choh Hao Li, Science 123, 617 (1956).

[8] V. N. Nikitin, R. L. Glubitskaya, O. P. Silin et al., Uchen. Zapiski Khar'kov. Univers. 24, 80 (1956).

[9] V. N. Nikitin, Priroda No. 2, 41 (1958).

[10] H. Gershberg, Endocrinology 61, 160 (1957).

[11] B. Baker, Dissert. Abstrs. 15, No. 5, 655 (1955).

[12] A. Segaloff, E. L. Komrad, A. Flores, and M. Hardesty, Endocrinology 57, 527 (1955).

[13] A. E. Wilhelmi, J. B. Fishman and J. A. Russell, J. Biol. Chem. 176, 735 (1948).

[14] Choh Hao Li, J. Biol. Chem. 211, 555 (1954).

[15] L. A. Eskin, Uspekhi Sovremennoi Biol. 46, 62 (1958).

[16] J. A. Russell and A. E. Wilhelmi, Annual Rev. Physiol. 20, 61 (1958).

[17] H. W. Larson, J. Biol. Chem. 94, 727 (1932).

[18] B. Fearon, Biochem. J. 38, 399 (1944).

[19] G. Schmidt and S. Thannhauser, J. Biol. Chem. 161, 83 (1945).

[20] V. Ya. Yur'ev, P. V. Kuchumov, G. N. Linnik, V. G. Vol'f and B. T. Nikulin, General Selection and Seed Technology of Field Cultures, p. 312, Cel'khozgiz [State Agricultural Press], M., (1950).

[21] D. K. Young and S. F. Cook, Proc. Soc. Exptl. Biol. and Med. 89, 482 (1955).

[22] E. V. Parina, Uchen. Zapiski Khar'kova. Univers. 25, 83 (1947).

[23] B. J. Gray, J. Endocrinol. 13, 429 (1956).

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THE EFFECT OF HEXOSE PHOSPHATES ON THE GROWTH AND SOME METABOLIC REACTIONS OF DYSENTERY BACTERIA AND COLON BACILLI

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Carbohydrates in microorganisms not only act as direct sources of energy, but also take part in the synthesis of intracellular polysaccharides [1-4]. A number of bacteria can grow on nutritive media in which the chief source of organic substances is glucose. In these cases glucose and the products of its transformation go to form different component parts of the microbial cell. In spite of the variety of the parts played by carbohydrates in metabolic reactions of microorganisms, the pathways of reaction of simple and complex sugars, especially in pathogenic bacteria, have not been studied sufficiently.

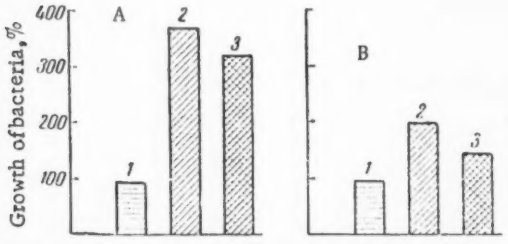

Fig. 1. Growth of dysentery bacteria depending on composition of the culture medium: A) Flexner bacteria; B) Sonne bacteria; 1) glucose; 2) glucose-1-phosphate; 3) glucose-6-phosphate.

In the present work we give data on a comparative study of the effect of glucose and its phosphates on the growth and some metabolic reactions of dysentery bacteria and colon bacilli.

METHODS

The work was carried out with Flexner dysentery bacteria (strain No. 170) and Sonne bacteria (strain No. 130), and with colon bacilli (strain No. 675). The bacteria were grown on a synthetic nutritive medium which contained per liter of distilled water: 1 g of $(NH_4)_2HPO_4$, KH_2PO_4 and NaCl, 0.1 g $MgSO_4$, 20 mg tryptophan, and 5 mg nicotinic acid. Glucose was added in the amount of 5 g per liter, and glucose-1-phosphate and glucose-6-phosphate correspondingly contained 5 g of glucose. The growth of the bacteria was determined by an optical standard of cloudiness of the State Control Institute. The intracellular polysaccharides were studied by treatment with periodic acid and the Schiff reaction. Respiration of the bacteria was studied in a Warburg manometric apparatus.

RESULTS

Figure 1 gives the results on the effect of glucose and the phosphates on the growth of Flexner and Sonne dysentery bacteria. The growth of the Flexner bacteria was 3-4 times (determined after 18 hours), and of the Sonne bacteria 1½-2 times (after 8 hours) more intense on a medium with glucose-1-phosphate and glucose-6-phosphate than on a medium with glucose (Fig. 1).

When fructose-1,6-diphosphate was added to the medium along with glucose, the growth of the bacteria was the same as in the samples with glucose, and in some experiments was slower. One of the possible explanations of this effect was that the presence in the molecule of two phosphate residues increased its dimensions

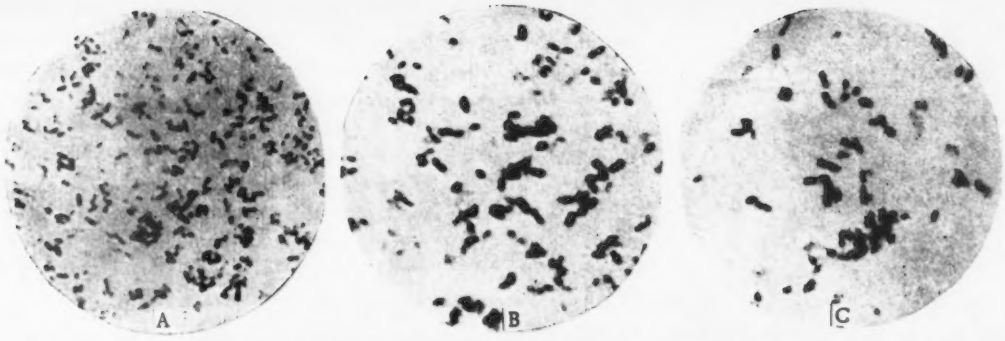

Fig. 2. Morphology of dysentery bacteria (Flexner) depending on composition of the culture medium: A) medium with glucose; B) with glucose-1-phosphate; C) with glucose-6-phosphate.

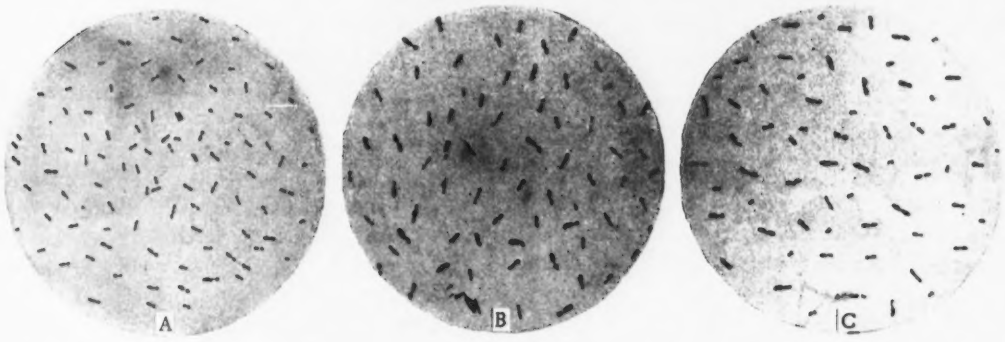

Fig. 3. Content of polysaccharides in dysentery bacteria (Flexner) depending on composition of the culture medium. Treatment with periodic acid and Schiff reagent: A) medium with glucose; B) with glucose-1-phosphate; C) with glucose-6-phosphate.

and hindered the entrance of fructose-1,6-diphosphate into the cell. To answer this question we studied the activity of aldolase in the whole microbe cell and on extracts from it. The experiments were carried out in the presence of bromoacetic acid. The findings showed that when fructose-1,6-diphosphate was added to the washed microbe cells there was splitting of 20-25% of the phosphorus, while the aldolase activity was sharply suppressed. In the experiments with extracts, splitting of phosphates occurred less energetically but the aldolase activity was higher. Thus, these results agree with the above suggestion of the reason for the weak growth of the bacteria studied in a fructose-1,6-diphosphate medium.

In Fig. 2 we give the morphology of the Flexner bacteria in a medium with glucose and with its phosphates. The figure shows that the size of the bacteria growing on media with glucose-1-phosphate and glucose-6-phosphate was greater than on a medium with glucose. These results show that the quantitative differences which were shown in Fig. 1 are reflected not only in the number of bacteria, but also in their size. Comparison of the results on the polysaccharide content in Flexner bacteria obtained in media with glucose and the phosphates (Fig. 3) showed that the quantity of polysaccharides in the cells was proportional to the size of the bacteria. Since the full development of bacteria of the intestinal groups depends in large measure on the polysaccharide components, we tested the virulency and immunological characteristics of these bacteria. The results showed that bacteria obtained from media with glucose and the phosphates, developed to the same degree of cloudiness had the same virulence and immunogenic power. It follows from this that the biological activity of the bacteria was proportional to their number and size.

In order to determine the intensity of metabolic processes in the studied bacteria during growth on a medium with glucose or its phosphates, we carried out the following experiments to determine the respiration of the corresponding cultures.

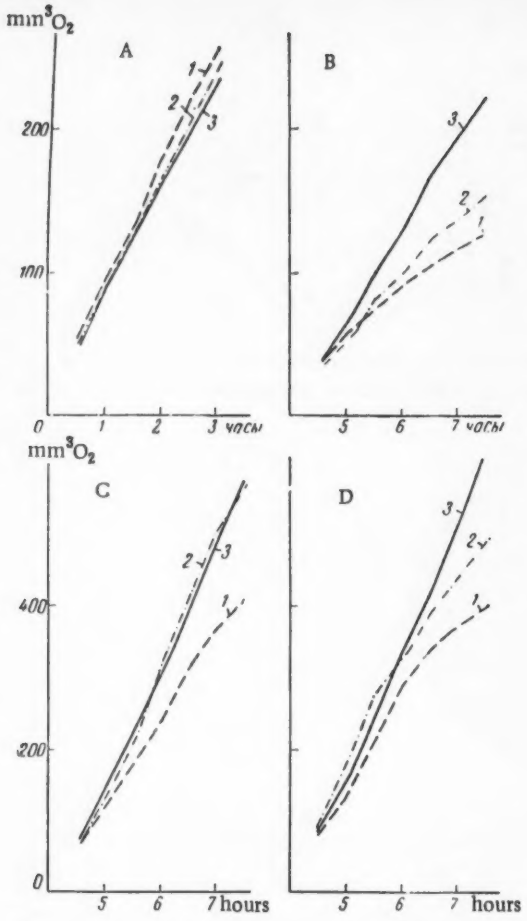

Fig. 4. Intensity of respiration of bacteria depending on growth of cultures and composition of the culture media: A) colon bacilli immediately after seeding; B) after 4 hours; C) Sonne bacteria; D) Flexner bacteria 4 hours after seeding. 1) medium with glucose; 2) with glucose-6-phosphate; 3) with glucose-1-phosphate.

The bacteria were seeded onto the media described above. In one series of experiments we measured the respiration beginning directly after seeding, in another, the culture was first kept in a thermostat for four hours at 37° and only after this was it transferred to the vessel of the Warburg apparatus for determination of the respiration. The bacteria were added at a value of 500 million per 1 ml of the nutritive medium. Measurement of the respiration was carried out for four hours at 37°.

In Fig. 4 we give the curves of oxygen absorption with growth of colon bacilli in media with glucose and the phosphates in the first four hours of growth, when oxygen absorption depends little on the form of the carbohydrate in the nutritive medium. However, with growth of the cultures, the picture changes (Fig. 4).

The oxygen requirement of dysentery bacteria and colon bacilli after a preliminary four hour keeping in a thermostat is increased considerably in media with glucose-1-phosphate and glucose-6-phosphate over that in a glucose medium. We must remark that the increased oxygen requirement occurs parallel with the growth and multiplication of the bacteria. Slower growth of the dysentery bacteria and colon bacilli in a glucose medium compared to a monophosphate medium must be assumed to be related to insufficient activity of the hexokinase of these bacteria.

These results also show that hexokinase activity limits the rate of transformation of glucose not only in the cells of multicelled organisms [5, 6], but also in bacteria.

SUMMARY

The growth of dysentery bacteria of Flexner and Sonne, and also of colon bacilli, is considerably faster on media with glucose-1-phosphate and glucose-6-phosphate than on media with glucose. It was observed that increase in the microbe mass depended not only on increased number of bacteria, but also on their size; the quantity of polysaccharides in the microbe cells was proportional to their dimensions; respiration of the bacteria was more intense in media with glucose-1-phosphate and glucose-6-phosphate than on a medium with glucose. The advantage of glucose-1-phosphate and glucose-6-phosphate over glucose is evidently related to insufficient activity of the hexokinase of these bacteria.

LITERATURE CITED

[1] S. J. Barker, Chim. Soc. 10, 2884 (1950).

[2] S. Levine, H. Stevenson, E. Tabor, R. Bardher and L. J. Chamber, Bacteriology 66, 664 (1953).

[3] V. I. Ivanov, A. K. Volchok and A. V. Lobanova, Biokhimiya 21, 760 (1956).

[4] V. I. Ivanov, Addresses to the Conference on Chemistry and Metabolism of Carbohydrates, Izd. AN SSSR, Moscow (1958).

[5] N. L. Belyaeva, B. P. Golovin, V. S. Il'in, O. N. Sytinskaya, T. V. Titova and M. P. Fomina, Addresses to the Conference on Chemistry and Metabolism of Carbohydrates, Izd. AN SSSR, Moscow (1958).

[6] S. A. Neifakh and M. P. Mel'nikova, Biokhimiya 23, 440 (1958).

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THE STIMULATION BY ATP OF PROTEIN SYNTHESIS IN LIVER HOMOGENATES DURING ONTOGENESIS

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The present work, like several published earlier [1-7], deals with the character and mechanism of the age-dependent decline of the protein-synthetic processes in the tissues and organs of the higher mammals. It was of particular interest to attempt to discover to what extent this decline in protein synthesis depended on the age-dependent change in the general central and inter-organ regulatory mechanisms, and to what extent changes in the actual biochemical processes and intracellular organization of the tissues were responsible. In the latter case, it seemed reasonable that one of the important factors would be the decreased ability of tissues to utilize efficiently the energy of various donors (viz. ATP) for the processes of protein synthesis.

The previous report from this laboratory [8] dealt with the effect of age on the synthesis of peptide bonds in liver and muscle homogenates, and with the stimulation of such synthesis by high-energy compounds. Using glutamine synthesis from glutamate and ammonium chloride as a test system, it was shown that the decreased ability to utilize the energy of high energy donors (ATP) may be the underlying reason behind the declining rates of peptide bond synthesis with age. Since the above conclusion was arrived at as a result of work on a simplified model system of peptide bond synthesis (glutamate + ammonium chloride $\rightleftharpoons$ glutamine), it was important to confirm this observation under conditions of net protein synthesis and its stimulation by ATP in homogenates of these tissues, and particularly of liver. The present paper deals with these experiments.

METHODS

Albino rats, aged 30, 90, 360 and 720 days were used. The animals were killed by decapitation and their livers removed as rapidly as possible and placed into a vessel cooled on ice. The tissue was homogenized with 10 volumes of 0.25 M sucrose. Samples of the homogenates (0.5 ml) were placed into previously prepared vessels containing all other additions, and ice-cooled. Vessel 1 (control) contained phosphate buffer, pH 7.4 and 0.5 ml of the homogenate.

Vessel 2 contained, apart from the basic contents in vessel 1, the amino acids tryptophan, arginine, threonine, lysine, leucine, valine, histidine, isoleucine, phenylalanine, tyrosine, methionine, glutamic acid, aspartic acid, alanine, proline, serine, cysteine and glycine. All amino acids were present in a final concentration of 0.2 mg/ml. The vessels also contained the following other additions: 0.1 ml of 0.3 M succinate, 0.25 ml of 0.3 M HCl, 0.1 ml of 0.198 M $MgCl_2$ and 0.1 ml of a solution of cytochrome c (0.6 mg of cytochrome).

Vessel 3 contained the same additions as vessel 2, with the further addition of 0.1 ml of a neutralized solution of ATP (45 mg of ATP in 1 ml of water and 3 drops of 10% KOH). The final volume in all vessels was brought to 4 ml. The mixtures were treated according to Khesin et al. [9, 10], with the exception that turbidity of protein solutions was avoided by washing the precipitates with hot, 96° ethanol. Protein was estimated by the Biuret reaction according to Kirk [11]. The difference between vessels 1 and 2 represented protein increase dependent on the presence of amino acids. The difference between vessels 2 and 3 presented the stimulation of protein synthesis by ATP. The statistical evaluation of the differences was made according to the method of coupled pairs [18].

The Effect of Age on the Rates of Protein Synthesis from Added Amino Acids, and in the Presence and Absence of ATP, in Liver Homogenates (Protein synthesis in mg/vessel. Mean values)

Number of animals	Age, days	Composition of test			Stimulation of synthesis by additive			
		homogenate	homogenate + amino acids	homogenate + amino acids + ATP	amino acids	ATP	amino acids, %	ATP, %
12	30	3,65	3,96	4,48	+0,31	+0,52	+8,49 (6,0)	+14,25 (8,8)
11	90	3,86	4,14	4,63	+0,28	+0,49	+7,25 (6,0)	+12,69 (4,3)
11	360	3,98	4,27	4,68	+0,29	+0,41	+7,29 (4,2)	+10,30 (8,0)
9	720	3,93	4,24	4,63	+0,31	+0,39	+7,88 (8,6)	+9,92 (10,0)

Note: Numbers in brackets denote the significance of the difference.

RESULTS

The results of the experimental series are presented in the table. Total protein content in the samples used, reflecting the actual condition in the native tissue, was found to increase with age. This could be expected on the basis of other work by Magornyi et al [12, 13].

The second, and rather unexpected observation, was the almost equal level of potential ability of the homogenates to synthesize protein. Thus, when amino acids and ATP were added, liver homogenates from rats of all ages showed very similar increases in protein upon incubation. The synthetic ability of the liver homogenates of one-month old rats alone was slightly higher than that of the remaining age groups. The increase in protein in these youngest animals was 8.49%, as compared with 7.25-7.88% in the animals of all remaining groups.

The results also showed that with increasing age the stimulatory effect of added ATP on protein synthesis in the liver decreased gradually. Thus, the addition of ATP produced a relative increase of 14.25%, 12.69%, 10.30% and 9.92% in protein synthesis in one-month, three-months, one-year and two-year old animals, respectively.

Thus, in the present work, unlike in that previously described, the decline with age of the stimulatory effect of ATP on protein synthesis was established under actual conditions of net protein synthesis, and not on a model system for peptide bond synthesis.

DISCUSSION

The fact that no significant age effect was observed in the ability to synthesize protein by liver homogenates, that is, after the loss of cellular structure, may be explained by a number of possibilities. Firstly, it may be assumed that the aging processes develop very slowly in the liver, since this organ is known to change very little with advancing age. The process of change with growth of any organism is essentially heterochronic, and the liver may be held as an example of relatively slowly aging organs (as also are intestine and red bone marrow) [1, 7, 14, 15].

On the other hand, the liver is an organ continuously engaged in synthesis of high intensity, comparable to the intense activity of, say, the pancreas of the salivary glands. It is therefore possible that the change with age in the synthesis of its own tissue proteins by the liver may be masked by the high level of functional synthesis directed towards the maintenance of blood protein levels.

It is also quite probable that in tissue homogenates, where the native structure of the cell is disrupted, conditions for synthesis are no longer as favorable as in the intact organ, or in slices. Although synthesis still persists in homogenates, [16-18] the higher levels possibly existing in the younger animals may not be detected

under the artificial conditions. It should, however, be borne in mind that the intensity of oxidation in such homogenates was quite high and showed a pronounced age effect [19]. It is also noteworthy that the actual values for protein synthesis under the present conditions were higher than with other techniques [16, 17]. With all the limitations, the finding of the high protein-synthetic ability of the tissues of old animals is of great interest. At an age when in the intact animal synthesis towards growth almost disappears, and the so-called stabilizing synthesis (the inverse of the value of the wear coefficient) is considerably lowered, the potential for protein synthesis in the cell cytoplasm (or, more accurately, in cell homogenates) of the liver persists on high levels.

The fact that, even under the conditions of considerable disruption of the vital structure of the cell, the age effect on the stimulation of protein synthesis by ATP could be demonstrated, is undoubtedly of interest.

SUMMARY

Protein synthesis in liver homogenates was found to be considerable even in old age, its intensity being only very slightly below that found in the liver homogenates of young animals. The stimulatory effect of ATP on protein synthesis by liver homogenates was found to decrease with increasing age.

LITERATURE CITED

[1] A. V. Nagornyi, The problem of Aging and Longevity. Khar'kov University Press (1940).

[2] B. N. Nikitin, Old Age Ukr. SSR Academy of Science Press, Kiev (1940).

[3] V. N. Nikitin, Fiziol. Zhur. SSSR 30, 619 (1941).

[4] V. N. Nikitin, Uchen. Zapiski Khar'kova. Gos. Univers. 25, 95 (1950); Trudy Inst. Biol. 12, 95 (1947).

[5] V. N. Nikitin, Zhur. Obshchei Biol. 9, 112 (1948).

[6] V. N. Nikitin, R. L. Golubitskaya et al., Proceed. of Sympos. on Effect of Age on Metabolism and Reactivity of Organisms, p. 17. Ukr.SSR Academy of Science Press, Kiev (1951).

[7] V. N. Nikitin, Trudy Inst. Biol. Khar'kovs. Gosud. Univers. 21, 29 (1954).

[8] V. N. Nikitin, Trudy Instit. Biol. Khar'kovs. Gosud. Universit. 21, 113 (1954).

[9] R. B. Khesin and S. K. Petrashkaits, Biokhimiya 20, 597 (1955).

[10] R. B. Khesin and S. K. Petrashkaits, Biokhimiya 22, 501 (1957).

[11] P. Kirk, "Quantitative Ultramicroanalysis." Foreign Literature Press (Moscow, 1952) [Russian translation].

[12] R. L. Golubitskaya, Trudy Instit. Biol. Khar'kova. Gosud. Univers. 5, 81 (1938).

[13] R. L. Golubitskaya, Uchen. Zapis. Kharkivsk. Derzh. Univers. No. 12, 197 (1938).

[14] E. V. Cowdry, Problems of Ageing, Williams and Wilkins Comp. Baltimore (1942).

[15] M. Burger, Altern und Krankheit, Veb. G. Thieme, Leipzig (1957).

[16] R. B. Khesin, Biokhimiya 19, 407 (1954).

[17] R. B. Khesin, Biokhimiya 18, 462 (1953).

[18] V. Ya. Yur'ev, P. V. Kuchumov, G. N. Linnik et al., The General Selection and Seed Technology of Field Cultures Sel'khozgiz, (Moscow, 1950).

[19] J. R. Mclean and G. L. Cohn et al., Biol. Chem. 233, 657 (1958).

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STABILITY OF SOME PEPTIDE N-GLUCOSIDES AND OF PROLINE N-GLUCOSIDE IN AQUEOUS SOLUTIONS

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We have investigated the properties of N-glucosides of peptides and amino acids, which may serve as models of carbohydrate – protein linkages present in some proteins. We showed, in an earlier paper [1], that glycylglycine N-glucoside is much more stable than are the corresponding glucosides of amino acids (serine and glycine). The object of the present research was to ascertain whether the greater stability of N-glucosides of peptides, as compared with those of amino acids, is a general property of such compounds, and, in particular, whether it extends to the glucosides of higher polyglycines. We also investigated the effect on the stability of the glucosides of the absence of a hydrogen atom from the glucosidic nitrogen atom (examined for the case of proline N-glucoside).

As in the preceding paper, hydrolysis of the glucosides was followed by polarographic analysis of the glucose content of the solutions.

METHODS

Preparation of Peptide N-Glucosides

The N-glucosides were synthesized by the method of Micheel and Klemer [2], with the previously described modifications [1]. The earlier procedure was varied in one respect; no special precautions against access of atmospheric humidity were taken during the purification of the glucosides, by redissolving them in absolute methanol, and reprecipitating with absolute ether, followed by separation of the precipitate on the centrifuge. This modification greatly simplified the preparation, although the analytical results showed the presence of a certain amount of water in the products. We synthesized the following N-glucosides:

Sodium DL-alanylglycylglycinate N-glucoside ($C_{13}H_{20}O_9N_3Na$)

Found %: C 40,49, H 6.10, N 10.41%; Calculated %: C 40.3, H 5.68, N 10.8.*

The N-glucoside underwent hydrolysis when chromatographed, using the solvent system butanol – acetic acid – water (4:1:5), giving two spots, due to glucose and to alanylglycylglycine. Alanylglycylglycine N-glucoside has not previously been described in the literature.

Sodium glycylglycylglycinate N-glucoside. Our preparation was a mixture of N-glucosides of tri- and tetra-glycine, with a small amount of diglycine N-glucoside, as was shown by the chromatograms given by it, showing three peptide spots of different intensity (Fig. 1).

Sodium polyglycine N-glucoside. Judging from the chromatogram, our preparation contained tetraglycine N-glucoside, with a considerable admixture of penta- and hexa-glycine glucosides, and traces of di- and triglycine glucosides (Fig. 1).

*These figures relate to a sample of sodium alanylglycylglycinate N-glucoside prepared with exclusion of atmospheric humidity. The material taken for our experiments resembled this in every way, except that it contained substantially more water.

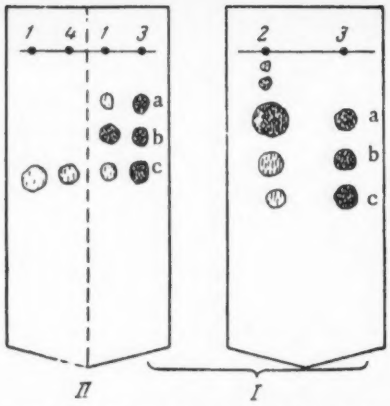

Fig. 1. Chromatograms of glycylglycylglycine ("triglycine") N-glucoside and polyglycine N-glucoside: 1) triglycine N-glucoside; 2) polyglycine N-glucoside; 3) mixture of peptides: a) tetraglycine; b) triglycine; c) diglycine; 4) glucose. I) Stained with tolidine; II) stained with aniline hydrogen phthalate.

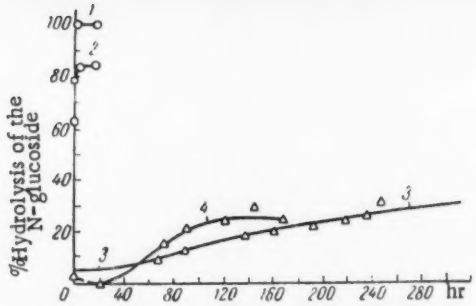

Fig. 2. Stability of alanylglycylglycine N-glucoside in solutions of different pH: 1) glucoside concentration 0.0191 g/ml, 0.1 N HCl; 2) 0.0202 g/ml, pH 5.3; 3) 0.0603 g/ml in water, pH 8.1; 4) 0.0412 g/ml, pH 9.7.

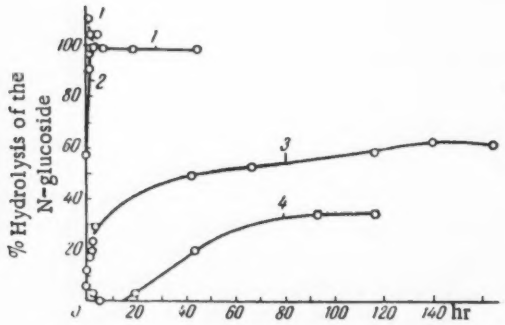

Fig. 3. Stability of "triglycine" N-glucoside in solutions of different pH: 1) glucoside concentration 0.0404 g/ml, 0.1 N HCl; 2) 0.0428 g/ml, pH 5.6; 3) 0.0406 g/ml, pH 7.6; 4) 0.0420 g/ml, pH 9.9.

Sodium DL-prolinate N-glucoside ($C_{11}H_{18}O_7NNa$). We were not able to separate our preparation from unreacted proline, and in view of its extreme instability we were obliged to use the crude preparation in our experiments. Judging from its glucose and amino group contents (determined by formol titration), it contained equivalent amounts of N-glucoside and proline.

Analysis of our preparation of proline N-glucoside gave the following results (in mg/ml):

Glucose found	NH found	Amount of glucose equivalent to that of NH found
7.98	1.20	15.5

No spots other than those due to glucose and proline appeared on the chromatograms.

Properties of the N-Glucosides Prepared by us

All the N-glucosides prepared by us gave a positive ninhydrin reaction on paper. In distinction from N-glucosides of amino acids, those of peptides do not give any colored products when heated at 100° for 5-10 min. or longer; characteristic brown spots are seen when the dry material is heated for 5 min at 170-175°. The component peptides and glucose do not under these conditions give any coloration when heated separately.

All the amino acid and peptide N-glucosides described in this and in our earlier paper [1] decolorize methylene blue in 0.1 N NaOH solutions after standing for some hours at room temperature.

Description of experiments designed to investigate the stability of the glucosides: a weighed portion of the N-glucoside was dissolved in the appropriate buffer, at room temperature (3 N acetate buffer of pH 5.5; 3 N NH_4Cl – NH_4OH buffer of pH higher than 7), or in water. The solution was placed in a polarimeter tube, and the optical rotation was read at regular intervals of time, at 20-21°. When the experiments were of long duration, we added a crystal of thymol to the solution. The degree of hydrolysis was calculated from the formula:

$$a, \% = \frac{[\alpha]_D - [\alpha_1]_D \, 100}{0{,}525\, b - [\alpha_1]_D},$$

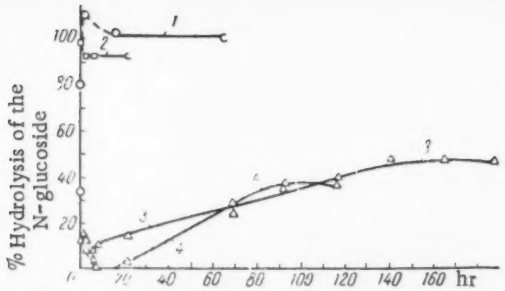

Fig. 4. Stability of polyglycine N-glucoside in solutions of different pH: 1) glucoside concentration 0.0169 g/ml, 0.13 N HCl; 2) 0.024 g/ml, pH 5.3; 3) 0.0508 g/ml in water, pH 8.6; 4) 0.0365 g/ml in water, pH 9.7.

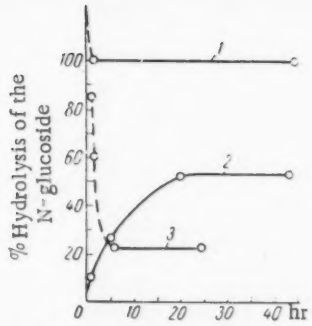

Fig. 5. Stability of proline N-glucoside in solutions of different pH: 1) glucoside concentration 0.0274 g/ml, 0.1 N HCl; 2) 0.0391 g/ml in water, pH 8-9; 3) 0.0375 g/ml, pH 9.9.

TABLE 1

Mutarotation of Glucose in 0.1 N HCl in the Presence of an Equivalent Amount of Glycine. Glucose – 0.0238 g/ml; 21-22°; l = 2.0 dm

Time elapsing from the moment of dissolution, hr	α	$[\alpha]_D$ of glucose
0,4	3,66±0,07	77,0±1,5
1,0	2,90±0,02	61,0±0,4
15,7	2,45±0,01	51,5±0,2
88,0	2,47±0,01	52,0±0,2

TABLE 2

Mutarotation of Glucose in Weakly Alkaline Solution in the Presence of Glycine. Glucose – 0.0060 g/ml; glycine – 0.0025 g/ml; $NaHCO_3$ – 0.003 g/ml; pH ~ 8.5; 22°; l = 1.0 dm

Time elapsing from the moment of dissolution, hr	α	$[\alpha]_D$
6,8	0,56±0,01	93±2°
32,1	0,32±0,01	53,3±2°

where a, % is percentage hydrolysis of glucoside, b is the percentage glucose content of the N-glucoside, as found by full hydrolysis with 0.1 N HCl, $[\alpha_1]_D$ is the lowest specific rotation found for the glucoside solution (this is found to be at pH 9.7-9.9), $[\alpha]_D$ is the specific rotation of the solution at a given moment, referred to the initial concentration of glucoside. In most cases, the values found for percentage hydrolysis during the first few hours of the reaction give only an approximate indication of the depth of hydrolysis, since mutarotation cannot be taken into account in calculating the results.

RESULTS

The following values of b and $[\alpha_1]_D$ were found:

N-glucoside of	b, %	$[\alpha_1]_D$
Alanylglycylglycine	41,0	—1,5°
"Triglycine"	37,6	—4,1°
Polyglycine	30,6	—1,7°
DL-proline	29,2	2,4°

The stability of the glucosides was measured at different pH values, and the results are illustrated by Figs. 2-5.

It is evident from the graphs that, under certain conditions, the values found for percentage hydrolysis of the glucosides appear to fall during the first few hours (this part of the curves is shown as a broken line in the graphs). This effect is due to a fall, rather than a rise, in positive rotation of the solutions. It may be ascribed

TABLE 3

Mutarotation of Glucose after Rapid Hydrolysis of Polglycine N-Glucoside in 0.13 N HCl. After hydrolysis the acidity corresponded to that of 0.1 N HCl. N-Glucoside – 0.0169 g/ml; 24-25°; l = 1.29 dm

Time elapsing from the moment of acidification of the solution, hr	$\alpha \pm 0.1$	$[\alpha]_D \pm 0.5$	% Hydrolysis	$[\alpha]_D$ of glucose, calculated on the basis of total hydrolysis of the glucoside (5.17 mg/ml)
0,2	0,13	6,0	33,8	19,5
0,6	0,31	14,2	80,0	46,5
1,6	0,39	17,9	110,5	58,5
15,3	0,36	16,5	103,0	54,0
64,0	0,35	16,0*	100,0	52,5

*It was found, on the basis of this result, that the glucose concentration when the glucoside was fully hydrolyzed amounted to 5.17 mg/ml.

TABLE 4

Mutarotation of Glucose after Rapid Hydrolysis of Alanylglycylglycine N-Glucoside in 0.1 N HCl. After hydrolysis the acidity corresponded with that of 0.06 N HCl. N-Glucoside – 0.0164 g/ml; 22– 20°; l = 1.0 dm

Time elapsing from the moment of acidification of the solution, hr	α	$[\alpha]_D$ of glucose, calculated on the basis of total hydrolysis of the glucoside (7.63 mg/ml)
2,5	0,89±0,03	117,0±4
28,9	0,57±0,02	74,6±2,6
46,6	0,39±0,01	51,1±1,3
74,2	0,41±0,03	53,5±4

to the fact that our glucosides belong to the α-series, and the glucose liberated on hydrolysis undergoes mutarotation. The tables shown above show that mutarotation of α-glucose corresponds closely with that of the glucose liberated during the rapid hydrolysis of glucosides in acid media (Tables 1 and 2) for glucose, and 3-5 for N-glucosides).

Our results show (Table 3) that α-glucose is plit off from the peptide glucoside in a short, but measurable, period of time, and is then rapidly converted into a mixture of isomers, owing to the high acidity of the medium.

The data of Table 4 show clearly that all of the liberated glucose has a high optical rotation, characteristic of α-glucose. Mutarotation proceeded in this experiment at a slower rate than in that of Table 3, since the acidity was lower.

The data of Table 5 show that when hydrolysis of the peptide glucoside proceeds very slowly, the fall in optical rotation over the first 5 hr due to mutarotation prevailed over the rise in rotation due to liberation of α-glucose.

DISCUSSION OF RESULTS

In the first place, we have to take into consideration the effects of secondary processes which may complicate the analysis of the polarimetric data for hydrolysis of N-glucosides. As is evident from the data presented, the substances synthesized by us are α-glucosides. On hydrolysis they liberate α-glucose ($[\alpha]_D$ = 110°),

TABLE 5

Changes in Optical Rotation of a Solution of Polyglycine N-Glucoside at pH 8.6 (in water). Sodium salt of polyglycine N-glucoside 0.0508 g/ml; 23-25°; l = 1.11 dm

Time elapsing from the moment of dissolution, hr	α	$[\alpha]_D$	Relative percentage hydrolysis, taking $[\alpha]_D$ of glucose to be 52.5°
0,3	0,05±0,01	0,9±0,2	14,7
0,9	0,03±0,01	0,5±0,2	12,4
2,5	−0,02±0,01	−0,4±0,2	7,32
4,9	−0,01±0,02	−0,2±0,4	8,5
5,6	0,01±0,02	0,2±0,4	10,7
19,5	0,05±0,01	0,9±0,2	14,7
67,5	0,14±0,02	2,5±0,4	23,7
91,2	0,26±0,02	4,6±0,4	35,5
115,3	0,30±0,00	5,3±0	39,4
139,2	0,38±0,01	6,7±0,2	47,4
163,4	0,38±0,01	6,7±0,2	47,4
187,3	0,37±0,01	6,6±0,2	46,8

which then undergoes isomeric transformation, to give an equilibrium mixture of α- and β-isomers, of $[\alpha]_D$ 52.5°. Hydrolysis of glucosides thus leads to a gradual rise in optical rotation, whereas mutarotation acts in the opposite direction. On the relative rates of these two processes will depend the shape of the polarimetric curves obtained, and hence also the values of the degree of hydrolysis calculated therefrom.

Hydrolysis of amino acid glucosides proceeds rapidly and smoothly, so that mutarotation does not exert much effect on the results, the rise in optical rotation due to liberation of α-glucose being many times greater. In acid media, in which the hydrolysis of N-glycosides of both peptides and amino acids proceeds practically to completion within a few minutes, the effect of mutarotation may be observed in an uncomplicated form (hydrolysis curves in 0.1 N HCl, Figs. 2-5). The same applies to hydrolysis of proline N-glucoside at pH 9.9 (Fig. 5): rapid hydrolysis is followed by approach to equilibrium within 30 min, after which the only effect seen is that of transformation of α-glucose to an equilibrium mixture of anomers.*

When the initial rate of hydrolysis of N-glucosides is very slow, as in alkaline media, the conditions for the process of mutarotation are such as to give the impression of an apparent fall in the degree of hydrolysis during the early stages of the reaction, immediately subsequent to dissolution of the substrate (the broken parts of the curves of Figs. 2-4).

For these reasons, our data for the rate of hydrolysis of peptide N-glucosides cannot serve as a basis for strictly quantitative treatment, and should be regarded as being of the nature of qualitative data, although the values found do not deviate very greatly from the true ones. This conclusion was drawn by us in our previous paper, from a comparison of the results of polarimetric analysis with those of formol titration of the liberated amino groups. The most trustworthy results appear to be those derived for the hydrolysis process at the stage of achievement of equilibrium, when the mutarotational process is also completed. The stability of different N-glucosides can therefore best be assessed from comparisons of the time required to attain equilibrium, and of the degree of hydrolysis achieved at this time. Some indication of the stability of the compounds can also be derived from comparisons of the slopes of the curves and from determinations of the degrees of hydrolysis over the short periods of time required for the dissolution of the substrates, although the values so obtained are no more than indicative. Our results for the hydrolysis of N-glucosides are illustrated by the curves of Fig. 6.

Like amino acid N-glucosides, peptide glucosides undergo rapid and complete hydrolysis to glucose and

*In this single case only, the observed fall in optical rotation, from 0.64° to 0.25°, did not correspond with full transition of specific rotation of glucose from 110° to 52.5°; theoretically, the change in rotation should have been from 0.48° to 0.25°.

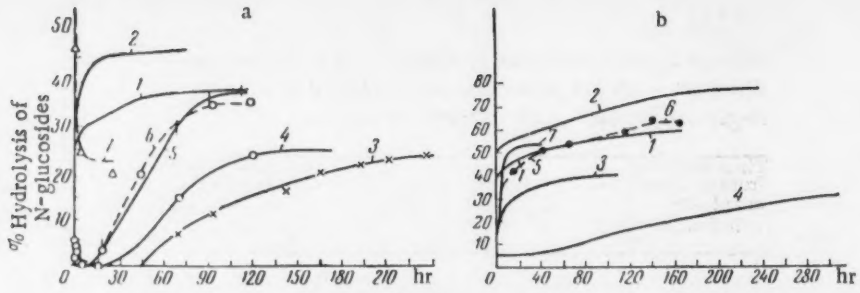

Fig. 6. Stability of N-glucosides of peptides and amino acids at pH 9.7-9.9 (a) and at pH 8 (b): N-Glucosides of 1) glycine; 2) serine; 3) diglycine; 4) alanylglycylglycine; 5) polyglycine; 6) triglycine; 7) proline.

peptides in 0.1 N HCl; as the pH of the solutions is raised, the composition of the equilibrium mixture is displaced progressively in the direction of N-glucoside. However, all the N-glucosides examined by us also undergo hydrolysis in 0.1 N NaOH, in which medium they gradually decolorize methylene blue, even at room temperature.

The degree of hydrolysis of peptide N-glucosides depends in the first place on the pH of the medium, and to a lesser degree on the nature of the amino acid or peptide aglucone. Peptide N-glucosides differ from those of amino acids in their slow rate of hydrolysis during the first 15-45 hr after dissolution in media of pH 9.7-9.9 (Fig. 6). Irrespective of the reasons for the distortion of the form of the curves during the first few hours of hydrolysis, the differences found between the curves for amino acid and peptide glucosides are very great, and are indicative of very great differences in the rates of hydrolysis of these two classes of glucosides. The greater stability of the N-glucosidic linkage of peptide glucosides is not, however, so great as to render it feasible to isolate such compounds from the hydrolyzates of naturally occurring products in any appreciable yield. Hence, the only promising procedure for establishing the presence of such N-glucosidic linkages with the α-NH_2 groups of N-terminal amino acids of proteins would appear to involve the raising of the stability of such linkages, by reduction of the glucosidic bridges, for example [3] (N-glucosidic linkages with the ϵ-NH group of lysine are quite stable, at least in acid media [4]).

A comparison of the effect of lengthening the peptide chain of a series of polyglycine N-glucosides on their stability at pH ~ 10 is of interest. The N-glucosides of "triglycine" and "polyglycine" are more stable than is that of glycine, but are less stable than is diglycine N-glucoside. No satisfactory explanation of the higher stability of the latter substance can be advanced as yet, but it is probably due to some specific feature of its structure. Comparison of two tripeptide glucosides – triglycine and alanylglycylglycine – shows that the properties of the N-glucosides are affected by the nature of the constituent amino acids of the peptides; introduction of alanine raised the stability of the glucoside. The stability of the linkage is raised by increase in the distance between it and the terminal carboxyl group of the peptide chain. The greatest effect is observed between amino acid and dipeptide glucosides. Further lengthening of the peptide chain to 3-4 units gives no further appreciable rise in stability. The nature of the amino acid in direct linkage with glucose has a greater effect on the stability of the glucosides of peptides.

Our experiments on the N-glucoside of proline showed that the presence of a tertiary cyclic nitrogen atom in the glucosidic linkage leads to a sharp fall in the stability of the product, the rate of hydrolysis of which is greater at all pH values than is that of other N-glucosides of amino acids and peptides. The degree of hydrolysis achieved at equilibrium was, however, within the limits found for amino acid glucosides at pH ~ 8, and for alanylglycylglycine glucoside at pH ~ 10.

SUMMARY

The stability of N-glucosides of peptides in weakly or strongly alkaline solutions is greater than that of amino acid N-glucosides. The difference is most clearly shown at pH ~ 10; amino acid N-glucosides undergo rapid hydrolysis in solutions of this pH almost from the moment of their dissolution, whereas those of peptides show no appreciable hydrolysis for several hours after being dissolved.

The composition of the equilibrium mixture of amino acid or peptide N-glucosides and their hydrolysis products depends in the first place on the pH of the solutions. At pH 9-10 it is displaced far to the left. Under such conditions, 25-35% of peptide N-glucosides were hydrolyzed at equilibrium, as compared with 37-46% for glycine and serine N-glucosides; at pH 8 the corresponding figures were 30-60% and 58-75%, respectively.

The stability of the N-glucosides rises with increase in the length of the peptide chain from glycine to triglycine; further lengthening of the polyglycine chain did not appreciably affect the stability of the glucosides. The N-glucoside of diglycine does not conform to this rule, as its stability exceeds that of the other members of this series; this may be due to some stabilizing factor associated with its molecular structure. Proline N-glucoside is distinguished by its exceptionally high rate of hydrolysis in both acidic and alkaline media.

LITERATURE CITED

[1] E. D. Kaverzneva and V. É. Kalis, Biokhimiya 23, 92 (1958).

[2] F. Micheel and A. Klemer, Chem. Ber. 85, 1083 (1952).

[3] F. Micheel and B. Scheeppinghoff, Chem. Ber. 89, 1702 (1956).

[4] F. Micheel and A. Klemer, Chem. Ber. 89, 1238 (1956).

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DIGESTIVE ENZYMES OF FISH AND SEASONAL CHANGES IN THEIR ACTIVITY

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The study of enzyme systems of poikilotherms, and in particular of the digestive enzymes of fishes, is of considerable importance to the development of comparative biochemistry. The fundamental research work on the enzymatic activity of the different levels of the digestive tract of fishes was done at the end of the 19th and the beginning of the 20th centuries [1]. The work was, however, mostly of a qualitative nature, and was based on randomly collected specimens. Comparison of the results is sometimes difficult. The number of published papers on seasonal variations in the activity of the digestive enzymes of fishes is very small. They include Pyatnitskii's work [2] on the variability of peptic activity in salmonoid fishes, Margolin's paper [3] on seasonal variability of tryptic and amylolytic acitivity in carp, and Kashiwada's paper [4] on seasonal variations in the activities of proteolytic enzymes of tunny fish.

The present paper deals with the results of a research on digestive enzymes (pepsin, trypsin, amylase and lipase) of burbot (Lota lota), pike perch (Lucioperca lucioperca), and bream (Abramis brama), from the Rybinsk reservoir. These species were chosen for study because they were the prevalent forms of fishes in the reservoir, and because they differed from each other in the structure of their alimentary tracts and in the nature of their food. Burbot and pike perch are carnivorous fish with well developed stomachs, while the bream is a typical benthophage fish, not possessing a stomach. Burbot feed most intensively during the winter months, while pike perch and bream feed most actively during the summer months. Our choice of animal material thus enabled us to relate the enzymatic activities of each species to its biological peculiarities.

Our research program included the assessment of the activities of pepsin, trypsin, amylase and lipase at different levels of the alimentary tract, the determination of the Michaelis constant of these enzymes, the investigation of the relations between enzyme activity and pH and temperature, and the measurement of the pH of the gastric and intestinal contents and the bile at the height of digestion.

METHODS

The fish were taken direct from the reservoir, and were fasted for 10 days. The length of the specimens varied from 30 to 40 cm. The tank temperature varied from 1 to 3° during the winter, 4 to 7° in the spring, 15 to 23° in the summer, and 8 to 10° in the fall.

The abdominal cavity of the live fish was rapidly exposed, and the stomach, hepatopancreas, pyloric processes, and foregut were carefully dissected out and immediately homogenized, with cooling. Pepsin was extracted with 0.2% HCl, and trypsin, amylase, and lipase by overnight extraction with 87% glycerol, with cooling. For extraction we took 200 volumes of solution per volume of tissue. The extracts were shaken energetically with ether, in a separating funnel, for 5 min, and the defatted lower layer was filtered on a Buchner funnel, through fabric and paper filters, successively. The transparent, faintly opalescent filtrates were diluted with an equal volume of water, and the resulting solution was taken for determination of enzyme activities. Amylase activity was determined by the method of Willstatter et al. [5], titrating the sugar produced by the method of Willstatter and Schudel [6]. The substrate was soluble starch, at a concentration of 0.790%. Peptic activity was assessed by the method of Rona and Kleinman [7]. The substrate used was 0.030% lactalbumin. The completeness of

TABLE 1

Activity of Digestive Enzymes of Fishes at Different Seasons

Month	Monomolecular reaction constant, in min^{-1}			
	pepsin	trypsin	amylase	lipase
Burbot				
February	0,0679	0,0291	0,0050	0,0232
May	0,0403	0,0129	0,0116	0,0348
July	0,0005	0,0090	0,0129	0,0057
October	0,0068	0,0084	0,0164	0,0051
Pike perch				
February	0,0014	0,0098	0,0205	0,0070
May	0,0280	0,0064	0,0046	0,0174
July	0,0420	0,0101	0,0064	0,0271
October	0,0050	0,0020	0,0245	0,0184
Bream				
February	0,0007	0,0088	0,0409	0,0035
May	0,0090	0,0123	0,0126	0,0319
July	0,0046	0,0129	0,0129	0,0251
October	0,0036	0,0063	0,0168	0,0177

TABLE 2

Michaelis Constants (I) and Maximum Velocities (II) of Enzyme Reactions
K_M is expressed as percentages, and reaction velocities as grams of substrate decomposed per second.

Month	Pepsin		Trypsin		Amylase		Lipase	
	I	II	I	II	I	II	I	II
Burbot								
February	0,024	0,006	0,040	0,059	0,563	0,040	0,144	0,002
May	0,033	0,004	0,050	0,040	0,861	0,096	0,067	0,002
July	0,026	0,002	0,130	0,040	0,463	0,086	0,209	0,001
October	0,019	0,004	0,130	0,037	0,430	0,073	0,302	0,001
Pike perch								
February	0,016	0,002	0,110	0,040	0,794	0,073	0,256	0,001
May	0,017	0,004	0,300	0,016	0,927	0,033	0,062	0,002
July	0,007	0,004	0,120	0,042	0,695	0,070	0,103	0,001
October	0,019	0,004	0,420	0,020	0,471	0,086	0,116	0,001
Bream								
February	0,044	0,004	0,120	0,031	0,132	0,073	0,481	0,001
May	0,009	0,005	0,180	0,051	0,596	0,040	0,084	0,002
July	0,017	0,004	0,060	0,036	0,596	0,040	0,086	0,001
October	0,026	0,003	0,140	0,029	0,851	0,063	0,212	0,002

precipitation of undigested protein was tested by the procedure of Derviz et al. [8]. A FÉK-54 photocolorimeter-nephelometer was used for the nephelometric measurements, with a No. 10 filter. The results were read from a previously constructed calibration curve. Tryptic activity was assessed by the method of Willstatter et al. [9], involving titration with 0.01 N alcoholic KOH. The substrate used was a neutral 0.16% casein solution. Lipase was assayed by the method of Balls et al. [10], titrating with 0.01 N alcoholic KOH. The substrate was a 0.060% solution of vegetable oil.

TABLE 3

Temperature Dependence of Digestive Enzymes of Fishes. Percentage of substrate decomposed

Species of fish and month of sampling	3 hours of incubation at a temperature of					10 hours of incubation at a temperature of				
	10	20	30	40	50	10	20	30	40	50
Pepsin										
Burbot										
February	18,4	28,8	42,0	80,0	2,0	26,0	93,6	82,8	80,0	5,0
May	8,8	11,6	43,0	73,6	1,2	18,0	72,8	80,0	77,6	2,0
July	1,0	3,0	6,8	19,6	0,2	2,0	28,8	40,0	29,0	1,6
October	7,2	10,8	19,6	40,1	3,2	9,8	40,0	58,0	56,0	4,8
Pike perch										
February	4,8	9,6	20,0	36,8	1,4	6,2	40,0	52,0	39,2	1,8
May	6,2	12,2	26,8	40,1	1,6	9,0	40,2	56,8	40,2	3,4
July	5,2	8,8	19,6	36,7	1,4	6,4	39,6	50,2	38,0	2,0
October	1,8	3,6	12,8	14,0	0,2	2,6	20,0	25,8	15,8	0,6
Bream										
February	2,8	7,6	20,0	26,0	1,6	3,2	29,0	30,8	32,0	1,6
May	16,6	23,0	50,0	86,0	2,6	19,0	70,0	83,2	88,0	3,0
July	15,2	20,0	40,0	60,4	2,0	16,0	68,8	81,6	62,4	2,0
October	6,8	14,8	20,1	52,0	1,6	8,0	40,0	58,0	52,0	4,2
Trypsin										
Burbot										
February	5,8	20,0	30,0	60,0	1,6	8,0	76,0	65,8	61,2	2,4
May	3,2	6,8	16,0	26,8	1,6	4,0	27,6	29,0	27,6	1,0
July	1,6	2,4	9,0	22,4	0,8	2,4	27,0	26,0	22,0	1,2
October	0,8	1,2	4,4	20,0	0,8	1,6	26,0	24,0	20,0	2,0
Pike perch										
February	12,8	16,0	36,0	40,0	0,6	16,0	58,0	46,8	42,6	0,8
May	14,8	16,0	40,6	49,6	0,2	17,0	58,0	51,2	49,6	0,2
July	12,0	19,0	40,0	46,8	1,6	16,8	58,2	52,2	48,0	3,4
October	2,6	5,2	16,8	20,8	0,2	4,8	26,2	36,0	34,0	0,2
Bream										
February	6,2	8,8	23,0	28,8	2,2	8,0	34,0	56,0	37,0	3,2
May	15,4	17,6	40,0	57,0	6,0	19,8	62,0	69,2	62,8	8,2
July	12,2	16,2	43,0	51,2	2,8	22,0	46,8	64,0	56,8	6,0
October	2,0	8,0	20,0	26,0	1,2	6,8	31,4	37,0	29,8	1,8
Amylase										
Burbot										
February	13,2	18,8	27,2	33,2	3,6	17,2	58,0	58,0	53,2	4,0
May	17,6	30,0	40,8	46,0	2,4	40,0	51,6	47,2	47,0	1,2
July	12,8	27,2	40,8	46,0	2,4	46,8	55,2	52,0	48,0	8,0
October	6,0	38,0	44,0	44,0	4,0	35,0	57,2	78,0	24,0	4,0
Pike perch										
February	14,8	18,2	36,8	41,6	1,2	21,2	46,8	62,2	45,0	8,6
May	7,2	10,8	21,6	24,8	4,2	12,8	36,8	40,0	26,0	4,4
July	13,8	16,8	30,0	40,6	8,2	18,0	50,6	59,8	42,6	10,0
October	19,2	36,8	51,2	60,2	12,2	27,6	73,0	61,0	60,0	12,4
Bream										
February	16,8	26,8	56,0	76,8	2,4	29,2	82,8	82,0	78,2	2,6
May	12,8	18,2	26,8	42,2	6,2	15,2	56,2	59,0	56,6	3,8
July	14,6	16,2	27,2	38,8	3,0	17,2	48,2	43,6	40,8	2,2
October	12,8	15,2	42,0	61,6	18,8	19,2	61,0	62,8	62,2	27,6
Burbot										
February	16,2	26,8	31,2	43,6	0,8	19,2	51,2	63,6	52,8	1,2
May	22,4	56,2	58,8	62,8	2,0	24,2	72,0	70,0	64,8	3,8
July	3,0	3,6	7,8	16,8	0,6	4,8	27,8	17,2	17,0	1,0
October	0,6	1,8	3,0	10,8	0,2	2,0	24,2	16,6	14,2	2,0
Pike perch										
February	0,8	1,2	3,6	20,0	0,4	2,0	26,2	25,0	24,0	1,0
May	11,8	46,8	62,2	66,0	2,2	20,0	76,8	68,2	68,0	2,4
July	6,8	28,6	51,2	56,2	6,0	10,2	55,8	63,0	60,6	6,2
October	5,0	26,0	40,0	50,0	1,0	12,0	56,0	56,2	52,8	2,4
Bream										
February	0,2	2,8	4,2	7,6	0,2	1,0	5,2	9,6	8,2	0,4
May	18,8	36,2	43,0	56,8	1,6	19,6	63,6	64,2	63,0	4,2
July	16,8	19,8	29,6	50,8	2,0	20,0	60,0	62,8	60,0	5,2
October	17,6	9,8	19,0	40,6	0,2	11,2	56,2	59,0	56,2	8,0

TABLE 4

Relation Between pH and Enzyme Activity. Activity is expressed as percentage of substrate decomposed during 3 hours of incubation at 30°

Month	Pepsin, pH			Trypsin, pH			Amylase, pH			Lipase, pH		
	1,5	2,0	2,5	6,5	7,0	7,5	6,5	7,0	7,5	7,5	8,0	8,5
Burbot												
February	10,2	26,2	25,2	16,8	40,0	11,6	17,6	28,0	18,2	19,6	31,2	17,2
May	8,6	16,0	31,0	13,0	20,0	10,2	15,8	42,0	31,6	24,8	58,8	31,2
July	0,4	0,6	1,6	1,4	18,2	0,2	22,4	40,6	17,2	5,0	7,8	3,6
October	2,3	11,5	12,3	0,6	4,4	0,8	27,2	42,3	16,3	2,0	3,0	2,2
Pike perch												
February	8,0	12,6	23,0	17,2	40,0	13,6	14,8	36,0	11,6	1,4	3,6	1,6
May	13,0	15,4	23,4	12,2	41,2	12,0	14,8	43,0	13,2	18,0	62,2	20,0
July	6,8	11,3	12,0	12,0	40,0	12,0	17,2	27,0	14,8	16,8	56,2	27,0
October	4,5	6,1	7,2	8,6	17,4	10,2	20,0	52,8	15,0	17,2	50,0	29,0
Bream												
February	4,3	13,3	24,1	18,0	24,0	11,2	19,6	57,0	17,2	2,0	7,6	2,8
May	16,7	24,3	41,8	17,8	45,2	12,4	13,2	40,0	12,8	27,2	36,8	17,2
July	13,7	33,8	42,3	11,3	56,0	12,0	14,0	21,0	17,0	20,0	35,8	18,8
October	10,8	12,4	15,6	11,6	20,0	11,4	11,6	42,0	14,2	16,0	40,8	11,4

All the enzyme systems were incubated for 10 min at 30°, with continuous electromagnetic stirring. The Michaelis constant, K_M, was derived from the relation between the initial velocity of the enzymatic reaction and the substrate concentration. The concentrations taken were: for pepsin, 0.006, 0.012, 0.018 and 0.024%; for trypsin, 0.020, 0.040, 0.060 and 0.080%; for amylase, 0.100, 0.200, 0.300 and 0.400%; for lipase, 0.030, 0.060, 0.090 and 0.120%. The initial velocity of the processes was derived from tangents to the curves expressing rates of degradation of the substrates, and the K_M values were derived from Lineweaver-Burk plots (of $1/v$ against $1/S$ [11]).

The temperature dependence of enzyme activity was examined by incubation at 10, 20, 30, 40 and 50° for 3-10 hr; activity was assessed on the basis of percentage degradation of the substrates, and an analysis of the reaction kinetics was not performed. The dependence of enzyme activity on pH was derived from the percentage degradation of the substrates after 3 hr of incubation at 30°. A LP-5 potentiometer was used for determination of the hydrogen ion concentration at different levels of the digestive tract of fishes.

The following numbers of individual fish were examined: winter, 14 burbots, 7 pike perch, 13 bream; spring, 14 burbots, 14 pike perch, 10 bream; summer, 7 burbots, 11 pike perch, 18 bream; fall, 12 burbots, 8 pike perch, 9 bream.

RESULTS

In our first series of experiments we investigated the distribution of the enzymes at different levels of the digestive tract. Peptic activity was found in the stomach and the pyloric processes, amylolytic activity in the hepatopancreas and the foregut, tryptic activity in the hepatopancreas, pyloric processes, and foregut, and lipolytic activity in the hepatopancreas. The activity of homogenates of the pyloric processes and foregut was, however, extremely small, as compared with those of the stomach and hepatopancreas. The foregut of the bream, which does not have a stomach, showed an unexpectedly high peptic activity.

TABLE 5

pH of the Contents of Different Parts of the Alimentary Tract of Fishes at Different Seasons of the Year

Month	Stomach	Foregut	Bile
	Burbot		
February	3,8	7,1	5,8
May	3,9	7,6	6,0
July	3,1	8,1	4,3
October	3,0	8,0	4,2
	Pike perch		
February	3,4	7,1	7,1
May	3,3	6,8	5,3
July	3,1	7,3	5,4
October	3,5	8,1	7,2
	Bream		
February	—	8,3	6,4
May	—	8,3	6,4
July	—	8,2	6,5
October	—	7,4	6,7

Since, under our experimental conditions, the enzymatic reactions were monomolecular ones, enzyme activity could be expressed by the corresponding constant, derived from the equation:

$$K = \frac{2,3}{t} \cdot \log \frac{A}{A - x},$$

where K is a constant, in min^{-1}, t is the reaction time, in min, A is the initial concentration of the substrate, and A - x is the final concentration of substrate [12].

The data presented in Table 1 show that the activities of the enzymes vary widely at different seasons of the year. The highest activities of pepsin, trypsin, and lipase are found during the period of intensive feeding; during the winter for burbots, and during the summer for pike perch and bream. Amylolytic activity, on the other hand, is highest during periods of almost total abstinence from food. The highest pepsin, trypsin, and lipase activities were found in the burbot, and the highest amylase activity in the bream. Peptic activity showed the greatest seasonal variations, and tryptic activity the least, being still quite high during periods of abstention from food.

The graphically derived values of K_M, expressed as percentages, do not correspond with those found for homeotherms [13]. The affinity of fish pepsin for its substrate is about 150 times greater than is that of mammalian pepsins, and of trypsin and lipase 14 and 30 times greater, respectively. The affinity of fish amylase for its substrate is, however, only about a third of that of mammalian amylases. It cannot hence be concluded that the emzymes of fishes and mammals are not identical, since the experimental conditions were not fully comparable. Such large differences between the K_M values are nevertheless noteworthy.

The highest maximum velocities (v) of the enzyme reactions were found for amylase, and the lowest for lipase (Table 2). The temperature dependence of the digestive enzymes of different species of fishes, at different seasons, was the same in all cases. Table 3 presents the results of experiments involving 3 and 10 hours of incubation. In this table, activities are expressed as percentages of the amount of substrate initially present which have undergone decomposition during the given species of fishes is considerably higher than is their body temperature (Table 3); it was found to be at 40° with incubation periods of 3 hours, while with 10 hours of incubation it fell to 30°, or even to 20°. The activity of the enzymes fell sharply at 50°, at which it was practically zero.

We also investigated the relation between enzyme activity and the hydrogen ion concentration of the solutions. The results of these experiments are presented in Table 4. As in the preceding table, enzyme activities are expressed as percentage of substrate decomposed during 3 hours of incubation at 30°. The following pH optima were found: pepsin, pH 2.0-2.5, trypsin, pH 6.5-7.0, amylase, pH 7.0-7.5, and lipase, pH 7.5-8.0. No appreciable seasonal or species differences were found for the pH optima. The pH optima of the digestive enzymes of homeotherms fall within the same ranges [14]. The pH values of the contents of the alimentary tract, measured on the third day after feeding, were slightly higher than the optimum pH values of all of the digestive enzymes, with the exception of lipase, for which the pH was optimal. The hydrogen ion concentration at the mucosal surface of the intestines and in the adjacent layers was, however, closer to the optimum for enzymatic activity [1]. The pH values found for the bile of the species examined was much lower than for mammalian bile, the pH of which rarely falls below 7.0 [1]. The pH of the contents of different parts of the alimentary tract is shown in Table 5.

It is evident from the table that the pH shows no substantial seasonal variations, and is much the same for all the species examined.

DISCUSSION OF RESULTS

The high activity of the digestive enzymes of fishes may be related to the circumstance that they swallow their food whole, without mastication.

Accumulation of reserves, chiefly in the form of fat depots, takes place during the intensive feeding period, which in the burbot falls during the winter months, and in the pike perch and bream in the summer. These reserves are practically all used up during the fasting period for the metabolic needs of the fish and for formation of gonadal products, in preparation for spawning. This seasonal rhythm would be expected to affect the activities of the digestive enzymes, as has been shown by the investigations of a number of workers [2-4], as well as by the results of the present research.

The coincidence of the maximum activities of pepsin, trypsin and lipase with the period of intensive feeding suggests that production of these enzymes in fish is related to the level of food intake, and for this reason should vary periodically. The retention of high tryptic activity during the fasting period, and the considerable rise in amylase activity at this time, suggest that trypsin, and in particular amylase, are in some way concerned in the mobilization and redistribution of reserve nutrients in fish. It is conceivable that these processes are achieved in the intestines of fish during the seasonal abstention from food. We do not agree with Margolin's opinion [3] that the high activity of trypsin and amylase found by him in carp during the winter season is ascribable simply to accumulation of these enzymes in the absence of digestive activity.

The presence of peptic activity in hydrochloric acid extracts of the foregut of the bream, which does not possess a stomach, is of special interest. Owing to the unfavorable pH, there can be no possibility of any proteolytic effect due to pepsin in this case. The significance of our finding is, therefore, not clear. It might be interpreted on the basis of Karpevich's concept [1] of the disappearance of peptic digestion in fishes with advance in the evolutionary scale. Karpevich's views are not, however, based on sufficiently firm evidence.

The differences between the values found by us for the K_M and the temperature dependence of the digestive enzymes of fish and the corresponding values for those of homeotherms cannot serve as firm evidence of the nonidentity of the digestive enzymes of these two classes of animals; such a conclusion could only be based on the results of experiments performed under identical conditions. Taking into account the change in the temperature optimum of the digestive enzymes of fish with prolongation of incubation time, it might be supposed that with still further prolongation to 3-8 days (the usual duration of digestion of food by fishes), the temperature optimum would fall to that of the body temperature of the fish. The digestive enzymes of homeotherms are, of course, inactive at such low temperatures.

The low activity of fish amylase found by us is in accordance with the findings recently announced by some Indian workers [15], that the activity of shark amylase is only half that of pig amylase.

There is very little seasonal variation in the temperature and pH dependences of the digestive enzymes of fish. This is evidence of the stability of their physicochemical properties, as proteins. The variations in the activities of the enzymes of the species examined are evidently due to seasonal variations in the process of elaboration of these enzymes.

SUMMARY

Seasonal differences were found in the activities of pepsin, trypsin, amylase and lipase of the burbot, pike perch, and bream, from the Rybinsk reservoir. Maximum activities were found for pepsin, trypsin and lipase of these species to coincide with periods of intensive food intake; during the winter months for burbot, and during the summer months for pike perch and bream.

The amylase activity of all three species of fish was at a maximum during the periods of almost total abstention from food, and of preparation for spawning. Tryptic activity remains at a high level during these same periods.

The Michaelis constants of the digestive enzymes of fish, and their temperature dependences, differ very considerably from those of homeotherms. Their temperature optima tend to fall progressively with increasing duration of incubation. Their pH optima fall within the same ranges as for the corresponding enzymes of homeotherms.

The seasonal variations in the activities of the enzymes are due to differences in their rates of production at different seasons.

LITERATURE CITED

[1] V. A. Pegel', Physiology of Digestion in Fishes, Tomsk State University Press, Tomsk (1950).

[2] N. P. Pyatnitskii, Collected Papers of the VI All-Union Congress of Physiologists, Biochemists and Pharmacologists, Academy of Sciences, Georgian SSR Press, Tibilisi (1937).

[3] G. M. Margolin, Transactions of the Voronezh Branch of the All-Russian Sci. Research Institute of Fish Husbandry, Vol. 3, Nos. 2 and 3. Oblast' Press (1940).

[4] K. Kashiwada, Bull. Japan. Soc. Scient. Fish. 18, 13 (1952).

[5] R. Willstatter, E. Waldschmidt-Leitz and A. R. F. Hesse, Z. physiol. Chem. 126, 143 (1923).

[6] R. Willstatter and G. Schudel, Ber. Chem. Ges. 51, 780 (1918).

[7] V. S. Asatiani, Handbook of Biochemical Methods (Laboratory Procedures), Gruzmedgiz, Tbilisi (1939).

[8] G. V. Derviz, M. N. Astrakhan and L. F. Vaksman, Biokhimiya 23, 1 (1958).

[9] R. Willstatter, E. Waldschmidt-Leitz, S. Dunaiturria and G. Kunstner, Z. physiol. Chem. 161, 191 (1926).

[10] A. K. Balls, M. B. Matlack and L. W. Tucker, J. Biol. Chem. 122, 125 (1938).

[11] H. Lineweaver and D. Burk, J. Amer. Chem. Soc. 56, 658 (1934).

[12] D. Neilands and P. Stumpf, Enzyme Chemistry (Foreign Lit. Press, Moscow, 1947). •

[13] E. Baldwin, Dyamic Aspects of Biochemistry, (Foreign Lit. Press, Moscow, 1949). •

[14] J. B. Sumner and G. F. Somers, The Chemistry of Enzymes and Methods in Enzymology, (Foreign Lit. Press, Moscow, 1948). •

[15] D. S. Chanekar, D. V. Bal and K. Sohoni, Proc. Indian Acad. Sci. Ser. A. 2, 43 (1956).

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THE EFFECT OF POTASSIUM ON AMYLASE AND LIPASE PRODUCTION IN PIGEON PANCREAS SLICES

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Pigeon pancreas slices are capable of synthesizing in vitro a number of enzymes, including amylase and lipase [1, 2]. The condition for the production of these enzymes has also been precisely studied and defined [3, 4].

The present work deals with the relative rates of amylase and lipase production in pancreas tissue slices and with the effect of potassium on the ratio of these rates. In a number of tissues, notably in brain, the potassium concentration in the suspending medium determines both the direction and intensity of some metabolic processes [5]. The effect of K^+ concentration on metabolic intensity has also been observed in subcellular particles. There is a remarkable increase in the rate of incorporation of amino acids into protein when the K^+ concentration in the suspending medium is increased [6].

In an attempt to explain the mechanism of K^+ effect on the synthesis of the two enzymes mentioned above, the interrelationship between the intracellular K^+ concentration and the K^+ concentration in the medium was also studied in the present work.

MATERIALS AND METHODS

Pigeons, starved 24 hours, were given intramuscularly 50-125 mg of carbamylcholine one hour before killing. Pancreas tissue was prepared for incubation according to Hokin [1]. The tissue slices (35-70 mg) were incubated aerobically, for 90 min at 40°, in 2 ml of Krebs-Ringer phosphate. The incubation medium contained 0.2% (w/v) of each glucose and casein hydrolysate ("Pronutrin"). In order to study the effect of K^+ concentration on the system, sodium salts in the basic medium were substituted by the respective potassium salts. The initial value of enzyme levels in the tissues before incubation were determined for each set of incubations. After incubation the tissues were ground in powdered glass, a known amount of the incubation medium was added and extracts were made for the assay of enzymatic activity.

Amylase was assayed according to Smith and Roy [7]. Soluble starch (Lakhema**) was used as a substrate. Color densities were read on a Coleman spectrophotometer at 620 mμ. Amylase activity was expressed in mg starch hydrolyzed at 37° by an enzyme preparation equivalent to 1 mg of fresh tissue.

Lipase activity was assayed manometrically by a modification of the procedure of Rona and Lasnitzki [8]. Tributyrin (Merck) was used as a substrate. The main compartment of each vessel contained 2.7 ml of tributyrin emulsion in Ringer's solution (the emulsion was prepared by the use of 1% solution of sodium oleate); the side arms contained 0.3 ml of the tissue homogenate diluted with Ringer's solution. The gas mixture contained 95% N_2 and 5% CO_2. The final substrate concentration was 1%. Lipase activity was expressed as μl of CO_2 evolved in 15 min at 37° per 1 mg of wet tissue.

Since the two enzymes investigated were produced a different rates, it became necessary to express the respective activities in initial and in modified incubations as relative values to the original activity of each

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enzyme in the tissue. The mean values for enzyme production in basic and modified incubation media, as shown in the figure, were expressed in the following units:

$$E = \frac{P_2 - P_1}{P_1} \times 100,$$

where E is the mean value of the results obtained, and P_1 and P_2 are the enzymatic activities in the basic and modified media respectively, in per cent of the original activity in the tissue.

Slices weighing 100-200 mg, incubated for 90 min at 40° in 3 ml of the medium described above, were taken for the determination of K^+ and Na^+ concentrations, moisture content, inulin space (i.e., space determined with inulin) and Q_{O_2}. The medium employed also contained 0.7% (w/v) inulin. Oxygen consumption was determined by conventional techniques using Warburg manometers. The effect of K ions was determined in media where K was completely substituted with Na^+. The initial ionic composition and moisture content of all tissues were determined before incubation. After incubation the slices were blotted with filter paper, weighed on a torsion balance and dried overnight at 95°. Water content was estimated as the difference between the weight of the wet tissue and the air-dried residue, and was expressed as ml H_2O per 100 g of dry tissue.

For Na^+ and K^+ assays, the ground dry samples were extracted for 5 days with 0.2 N H_2SO_4, and the extracts used for flame-photometric estimations. The results were expressed as meq K^+ (Na^+)/kg wet tissue.

Inulin determinations were made on a portion of the 0.2 N H_2SO_4 extract, using the method of Heyrovsky [9]. In the incubation medium inulin was determined after the precipitation of protein with TCA. Colorimetric readings were made with a Koutski spectrophotometer at 530 mμ. Inulin space was expressed as ml per 100 g wet tissue. The relative intracellular cation concentration was calculated on the basis of their known total tissue concentration and of the inulin space. Thus, if E is the inulin space in ml/kg wet weight of tissue, and H_2O_T tissue moisture in ml H_2O/kg wet weight (calculated from the content of ml/100 g of dry tissue), then the intracellular moisture content, in ml/kg of wet weight of tissue is given by:

$$H_2O_I = H_2O_T - E.$$

The intracellular K^+ concentration is given by:

$$[K^+]_I = \frac{K_T^+ - K_E^+}{H_2O_I} \times 1000,$$

where K_T^+ is meq K^+/kg wet tissue, while $K_E^+ = [K^+]_0/1000 \times E$, if K_E^+ is meq K^+ in the inulin space and $[K^+]_0$ is the K^+ concentration in meq/kg of the incubation medium.

All the results quoted have been analyzed statistically by the binary pair method.

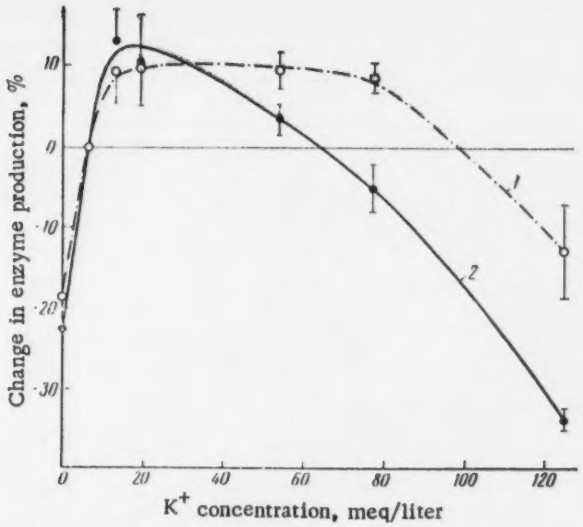
RESULTS

The Effect of K^+ on Amylase and Lipase Production

It was found (cf. Fig.) that alteration of the K^+ concentration affected the synthesis of both lipase and amylase. The shapes of the curves produced by the two enzymes are similar, but at certain given K^+ concentrations there were considerable differences in the rate of enzyme productions. When K^+ was fully replaced by Na^+ in the incubation medium production of both amylase and lipase declined considerably.

The optimal K^+ concentration for the production of either of the enzymes was higher than the K^+ content of the basic medium. When the K^+ concentration was raised (to 13 meq/liter), lipase synthesis increased very markedly; the rate of synthesis then remained unchanged over a wide range of K^+ concentration (up to 77.5 meq/liter).

Amylase production was found to react with greater sensitivity than lipase to alternations in K^+ concentration: the rate increased sharply when the K^+ concentration rose from zero to 13 meq/liter and gradually fell when the K^+ concentration was further increased. In the range of K^+ concentration 54-77.5 meq/liter the level of amylase production was approximately equal to, or slightly lower than, that observed in the basic medium.



The effect of potassium ion concentration in the incubation medium on the production of amylase and lipase by pigeon pancreas slices. (As a base line of 100% was taken the production of the enzymes in Krebs-Ringer phosphate – K^+ concentration 6.25 meq/liter): 1) lipase synthesis; 2) amylase synthesis.

With a K^+ concentration of 77.5 meq/liter in the incubation medium there was a considerable difference in the activity of the two enzymes. It was also found that very high K^+ concentrations (125 meq/liter) inhibited the production of both amylase and lipase.

In order to ensure that the observed effects of K^+ concentration in the incubation medium on the production of the two enzymes was not an artefact produced by the effect of K^+ on the actual enzyme assay systems, the effect of K^+ on the assay of both amylase and lipase were investigated. The results of those experiments proved conclusively that, at the concentrations employed in the incubation medium, K^+ did not affect the two enzyme assay procedures.

The Effect of Potassium on Respiration, Tissue Moisture, Inulin Space, and Cation Content

The effect of K^+ ion concentration on the production of the two enzymes, as established above, raised the question of the mechanism of this effect. Available information about the effect of this ion on the metabolic activity of tissues and cells allows the following assumptions to be made:

1) potassium ions may influence the aerobic metabolism and Pasteur effect in tissues, as was demonstrated by Ashford and Dickson [10] in brain cortex;

2) K^+ ions may cause a change in the intracellular content of K^+ and alter the cellular water metabolism [5].

These assumptions were tested experimentally, and the results are shown in Table 1. It will be seen from the table that alteration of K^+ concentration in the incubation medium had no marked effect on the value of Q_{O_2}. The tissue moisture content increased steadily with increasing K^+ concentration in the medium. However, high degree of hydration of the slices took place only when K^+ concentration reached 125 meq/liter. At that stage incubation conditions had no effect on the inulin space. Thus, even with considerable tissue hydration, the value of the inulin space remains constant. Pappius and Elliott [11], using similar methods, found that the extracellular space of brain slices, incubated in a medium containing 100 meq K^+/liter, remained virtually unaltered. Robinson [12] and Whittam [13] also reported the constancy of the inulin space in kidney slices under various degrees of tissue hydration.

The Effect of Potassium Ion Concentration in the Incubation Medium on the Oxygen Consumption, Inulin Space, Moisture, and Na^+ and K^+ Content of Pancreas. (Data represent means of 6 experiments. Incubation medium contained: 3 ml of physiological phosphate solution, 0.2% glucose, 0.2% casein hydrolysate and 0.7% inulin [w/v])

Characteristic	Initial value	K^+ concentration, meq/liter			
		6,25	13	54	125
Q_{O_2}	—	16,3±0,4	16,5±0,4	16,5±0,9	17,4±1,5
H_2O, ml/100 g dry tissue	279±7	291±7	304±7	308±7	428±10
Inulin space ml/100 g wet tissue	—	13,8±1,1	15,5±1,3	13,6±1,2	13,1±1,6
K^+, meq/kg wet tissue	117±6	102±2	90±2	110±6	139±5
Na^+, meq/kg wet tissue	43±2	57±4	66±3	45±5	23±4
$[K]_I$, meq/kg intracellular H_2O	—	159±3	144±2	168±11	182±6
$[Na^+]_I$, meq/kg intracellular water	—	66±7	79±3	57±4	32±2
$[K^+]_I/[K^+]_o$	—	25,4±0,5	11,1±0,2	3,1±0,2	1,4±0,1

According to published results [14], pancreas slices cannot maintain initial potassium (meq K^+/kg wet weight) values of the intact tissue. It can also be seen from the table, that the tissue slice potassium content, after incubation in a medium containing 13 meq K^+/liter, was even lower than in slices incubated in the basic medium. The original tissue potassium levels could only be maintained when the slices were incubated in media containing at least 54 meq K^+/liter. Original levels of tissue potassium in the slices were exceeded when the slices were incubated in the medium containing 125 meq K^+/liter. The changes in Na^+ concentrations were opposite to those of the potassium concentration.

Using the measurements of the inulin space, tissue moisture, and of the K^+ and Na^+ concentrations, it was possible to calculate the relative intracellular concentration of these ions (table). As the K^+ concentration in the medium increased, the intracellular concentration of this ion fell, while the intracellular concentration of Na^+ increased. When the extracellular potassium concentration reached 54 meq/liter, the intracellular concentrations of the two ions investigated approached the values found in incubation in the basic medium (the difference found lacked significance). As the K^+ concentration in the medium rose, so did the intracellular concentration of this ion, at the expense of the sodium ion. The ratio of intracellular to extracellular K^+ concentrations decreased with increasing K^+ concentration in the medium over the entire range of K^+ concentrations employed.

DISCUSSION

The results showed that amylase and lipase production in pancreas slices decreased considerably if potassium ions were either absent from the suspending medium, or were present in excessive concentrations. As in the case of glycogen synthesis in brain slices [5], definite K^+ concentrations are optimal for the synthesis of the enzymes mentioned.

It has been shown by a number of authors [15, 16] that potassium ions affect the cellular energy metabilism and that, at high concentrations, these ions activate the breakdown of ATP and phosphocreatine. This effect causes a lowering of the general levels of high energy phosphate in the cell and may affect the metabolic processes connected with energy utilization. It is therefore possible that enzyme production in the pancreas, being an endergonic and dinitrophenol-sensitive process, is inhibited by the high K^+ concentration in the medium. It is also possible that the resulting increased level of tissue hydration which causes a dilution of other cellular elements, of enzyme systems and substrates, aids the depression of synthetic activity.

The results also showed that a certain given K^+ concentration in the suspending medium affected the

synthesis of amylase and lipase in the slices to different extents. The highest difference was observed at a medium K^+ concentration of 77.5 meq/liter, when lipase synthesis was on a level considerably higher than that obtaining in the basic medium, while amylase synthesis was well below the corresponding value for this enzyme. According to Szabo and Garzo [17], amylase production in pancreas slices must not be considered as a de novo synthesis, but should rather be considered as a transformation from an existing, and as yet unidentified, precursor. There is little likelihood that K^+ ions are involved to a differential degree in the formation of peptide bonds in the synthesis of the two enzymes or of their precursors; it is therefore possible that potassium ions activate to a different degree the transformation of each enzyme precursor into the active form.

Krebs, Eggleston and Turner [14] found that pancreas tissue was unable to maintain K^+ levels during incubation (about 20-30% of the total ion concentration in the tissue was lost). The present results confirm this conclusion: even with an elevated K^+ concentration in the medium (13 meq/liter) potassium is still lost from the tissue. It cannot be argued that enzyme production is directly dependent on the intracellular K^+ concentration – at a medium K^+ concentration of 13 meq/liter, when the intracellular potassium was at a minimum, enzyme production was found to be maximal. However, amino acid incorporation into cellular structures takes place rapidly at high K^+ concentrations, and this indicates the importance of this metal in protein synthesis. In the authors' opinion, the important fact in the observations is the decreasing ratio of intracellular to extracellular potassium concentration with increasing K^+ concentration in the medium. The present results are in agreement with those of Choh-Luh Li and McIlwain [18] who found that partial substitution of medium Na^+ with K^+ lowered the resting potential of membranes of several isolated tissue preparations. It is quite possible that the most rational factor in metabolic processes is an absolutely defined ratio between the intracellular and extracellular potassium.

SUMMARY

The dependence of amylase and lipase production, in pigeon pancreas slices, on the K^+ concentration in the suspending medium was investigated. The synthesis of these enzymes reached a maximal level when medium K^+ was about 13 meq/liter. Further increase in K^+ concentration in the medium brought about a progressive fall in amylase production compared to lipase production. Oxygen consumption by the slices was not affected by the increased K^+ concentration in the medium.

Considerable tissue hydration took place at K^+ concentration in the medium of 125 meq/liter. In pancreatic slices "inulin spaces" of 13-15 ml/100 g wet tissue were observed; the value of this space did not depend on the degree of tissue hydration. With increasing K^+ concentration in the medium there occurred concomitant changes in the intracellular sodium and potassium concentrations and, in particular, in the ratio $[K^+]_I/[K^+]_0$.

LITERATURE CITED

[1] L. E. Hokin, Biochem. J. 48, 320 (1951).

[2] R. Schucher and L. E. Hokin, J. Biol. Chem. 210, 551 (1954).

[3] L. E. Hokin, Biochem. J. 50, 216 (1951).

[4] L. Rykhlik, G. Shveitsar and F. Shorm, Doklady Akad. Nauk SSSR 104, 283 (1955).

[5] A. Kleinzeller and R. Rybová, J. Neurochem. 2, 45 (1957).

[6] H. Sachs, J. Biol. Chem. 228, 23 (1957).

[7] B. W. Smither and J. H. Roe, J. Biol. Chem. 179, 53 (1949).

[8] P. Rona and A. Lasnitzki, Biochem. Z. 152, 504 (1924).

[9] A. Heyrovsky, Clin. chem. acta 1, 470 (1956).

[10] C. A. Ashford and K. C. Dixon, Biochem. J. 29, 157 (1935).

[11] H. M. Pappius and K. A. Elliott, Canad. J. Biochem. Physiol. 34, 1007 (1956).

[12] J. R. Robinson, Proc. Roy. Soc. B137, 378 (1950).

[13] R. Whittam, J. Physiol. 131, 542 (1956).

[14] H. A. Krebs, L. V. Eggleston and C. Terner, Biochem. J. 48, 530 (1951).

[15] P. J. Heald, Biochem. J. 57, 673 (1954).

[16] J. C. Skou, Biochim and biophys. acta 23, 394 (1957).

[17] M. T. Szabo and T. Garzo, Acta physiol. Akad. scient. hung. 12, 303 (1957).

[18] Choh-Luh Li and H. McIlwain, J. Physiol. 139, 178 (1957).

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α-1,4- AND α-1,6-POLYGLUCOSIDASES OF ANIMAL TISSUES

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Two types of α-1,4-glucosidases catalyzing the hydrolysis of glycogen and starch are now known. One of these types includes the α-amylases, which are endo-enzymes, splitting glycogen and starch molecules into large fragments, which then undergo further degradation. The second type includes the β-amylases and the γ-amylases (or glucamylases), which are exo-enzymes. The β-amylases catalyze the splitting off of maltose units from the nonreducing end of polyglucoside chains, while the glucamylases split off glucose.

α- and β-amylases are widely distributed among the higher plants, and α- and γ-amylases among yeasts and molds [1].

Until recently, only one α-1,4-polyglucosidase catalyzing the hydrolytic fission of starch and glycogen was known to be present in animal tissues, namely α-amylase. The presence of β-amylase in the pancreas, postulated by the Australian worker Goulston [2], has not been confirmed. Miller and Copeland [3] have recently reported the presence of a polyglucosidase of the γ-amylase type in beef prothrombin. Thus, various pathways of hydrolytic fission of polyglucosides such as starch and glycogen are present in animals as well as in plants.

The liver has been shown to contain not only α-1,4-, but also α-1,6-polyglucosidase, namely α-1,6-dextranglucosidase, which is an exoenzyme which splits off glucose residues from dextrans, up to the points of branching [4, 5]. It was found that preparations of liver dextranglucosidase, not exhibiting α-amylase or phosphorylase activity, attack glycogen as well as dextrans. The products formed from glycogen were dextrins, resembling β-dextrins in the absorption spectra of their iodine complexes, in their molecular weight range, and in a number of other properties [6]. The possibility that the liver enzyme preparations were contaminated with enzymes derived from blood, such as that of Miller and Copeland, was excluded by preliminary perfusion of the liver with physiological saline [6].

Dextranglucosidase may be a single enzyme, attacking both terminal α-1,4- and α-1,6-linkages in differently constructed polyglucoside molecules, or it may be a mixture of two different enzymes, one attacking terminal α-1,6-linkages of polyglucosides of the type of dextran (α-1,6-dextranglucosidase), and the other α-1,4-linkages of glycogen molecules (α-1,4-polyglucosidase, not identical with α-amylase). With the object of settling this question, we investigated the conditions under which glycogen and dextran are hydrolyzed by preparations of liver enzyme, and we attempted to achieve a separation of the two enzymatic activities. Degradation of glycogen and dextran was effected at different pH values and temperature, in the presence of inhibitors and sugars, and we applied differential fractionation with alcohol to separation of the activities, as well as fractionation by ion exchange chromatography and by starch slab electrophoresis.

EXPERIMENTAL METHODS

The preparation and purification of the liver enzyme has been described in an earlier paper [7]. The activity of the preparations was assessed on the basis of the amounts of reducing sugars produced during

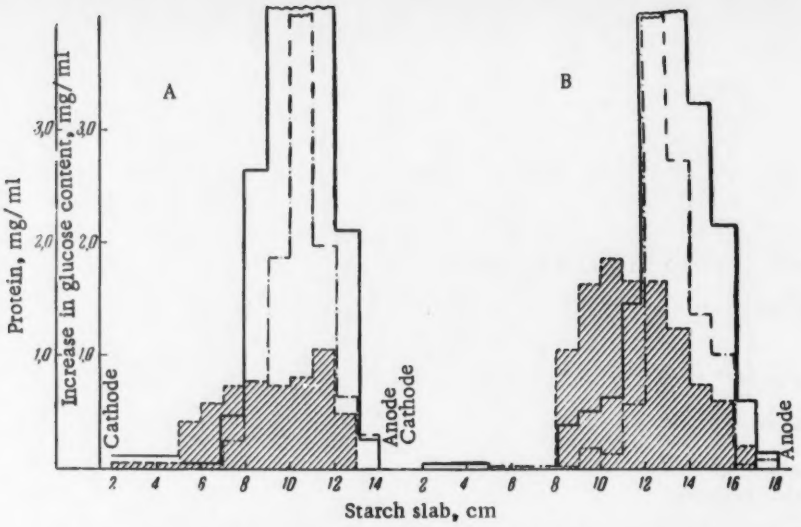

Fig. 1. Electrophoretic separation of α-1,4- from α-1,6-polyglucosidase, in a purified spleen enzyme preparation in starch gel: A) 0.05 M acetate buffer of pH 4.8; B) 0.05 M medinal hydrochloride buffer of pH 7.8. Continuous line — glycogen; broken line — dextran; shaded areas — protein. The arrows point to the point of application of the sample.

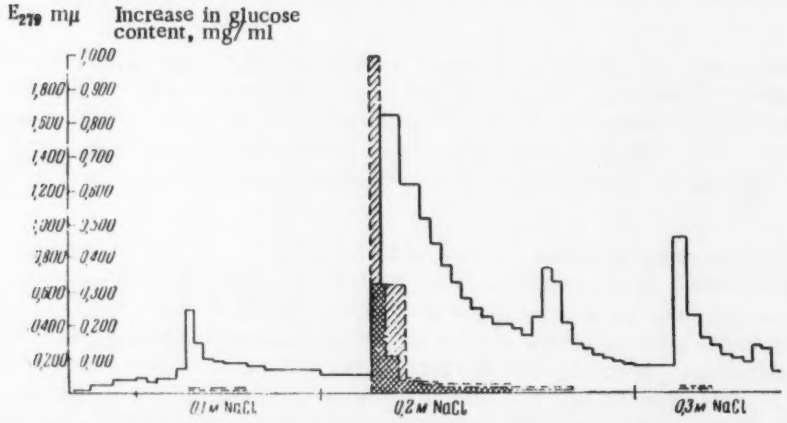

Fig. 2. Separation of α-1,4- and α-1,6-polyglucosidase activities of a liver enzyme preparation, on a carboxymethylcellulose column. Unshaded areas — protein; shaded areas — hydrolysis of glucogen; cross-hatched areas — hydrolysis of dextran.

incubation with glycogen and dextran. Their protein content was determined by Lowry's method [8]. Isomaltose was prepared by the method of Bacon and Bacon [9]. Intradex, manufactured in England, was taken as the dextran substrate. Glycogen was prepared from rabbit liver. A solution of histidine hydrochloride, of pH 4.8, was added to the enzyme preparation, to a final concentration of 0.1 M, and the mixture was left in the cold for 30 min. For the control systems we used the same enzyme preparation, without preliminary histidine treatment. p-Chloromercuribenzoate was dissolved in 0.1 N NaOH, the pH of the solution was adjusted to 7.4, and the resulting solution was added to the enzyme preparations, to a final concentration of $5 \cdot 10^{-5}$ M. The pH of the mixture was adjusted to 4.8 after 30 min. The control for this experiment contained the same enzyme preparation, the pH of which was adjusted first to 7.4, and then to 4.8 after standing for 30 min.

TABLE 1

Effect of Activity of α-1,4- and α-1,6-Polyglucosidases of Storage at Different pH. Incubation at pH 4.8 for 24 hours

Substrate	Increase in content of reducing substances, as mg/ml of glucose	Dextran / Glycogen, %
Storage at pH 3.5		
Glycogen	0	—
Dextran	0	
Storage at pH 4.8		
Glycogen	3,20	66
Dextran	2,12	
Storage at pH 7.0		
Glycogen	1,66	55
Dextran	0,92	

TABLE 2

Effect of the Activity of α-1,4- and α-1,6-Polyglucosidases of Incubation at Different pH. Storage at pH 4.8; 3 hr incubation

Substrate	Increase in reducing sabstances, as ml of glucose	Dextran / Glycogen, %
Incubation at pH 4.8		
Glycogen	0,895	54
Dextran	0,480	
Incubation at pH 7.0		
Glycogen	0,070	62
Dextran	0,030	

TABLE 3

Effects of Isomaltose and Glucose on Enzymatic Hydrolysis of Glycogen and Dextran. The figures in parentheses represent percentages.

Substrates	Increase in content of reducing substances, as mg of glucose per ml
Isomaltose	0 —
Glycogen	0,620 (100)
Glycogen + isomaltose	0,255 (41)
Dextran	0,355 (100)
Dextran + isomaltose	0,195 (55)
Glycogen	0,187 (100)
Glycogen + glucose	0,095 (50)
Dextran	0,162 (100)
Dextran + glucose	0,109 (66)

Fractional precipitation with alcohol was effected in the following way. The enzyme preparation obtained after three successive precipitations with ammonium sulfate (30-50% saturation) was heated at pH 4.8 at 55°, the protein precipitate was separated on the centrifuge and discarded, the proteins remaining in the supernate were precipitated with ammonium sulfate, and the precipitate was dialyzed against acetate buffer of pH 4.8. The resulting solution was fractionally precipitated with alcohol. Three fractions were thus obtained, at the following alcohol concentrations: 20, 20-30 and 30-50%.

Electrophoretic separation of the proteins was done on starch slabs, using 0.05 M acetate buffer of pH 4.8 or 0.05 M medinal buffer of pH 7.8. Electrophoresis was conducted in a cold room for 16-18 hr, with a potential gradient of about 22 V per cm and a current strength of 30-40 ma.

After conclusion of electrophoresis, the starch slab was cut into sections, from which the enzyme proteins were extracted with 0.2 M acetate buffer, in the cold. The protein solution obtained from each section was analyzed for protein, and its activity was assessed from the amount of reducing sugars produced during incubation with glycogen or dextran.

Separation of proteins by means of ion exchange chromatography was done on a carboxymethylcellulose column [10]. The enzyme solution was dialyzed against acetate buffer of pH 4.8. The dialyzed solution was then passed through the column. The effluent was collected in a fraction collector. The proteins were eluted from the column by 0.1 to 0.3 M NaCl solutions. The protein content of the eluates was determined by Lowry's method and from the optical density at 279 mμ. The enzymatic activity of the eluates towards glycogen and dextran was measured in the usual way.

RESULTS OF EXPERIMENTS

Fractionation of Enzyme Preparations

Fractional precipitation with alcohol. Three enzyme fractions were obtained. The activity of these fractions (increase in content of reducing substances, expressed as mg of glucose per ml) was determined at the same time, and under identical conditions. The results were as follows:

Alcohol concentration of fraction, %	Glycogen	Dextran	Dextran / Glycogen, %
20	1.90	1.10	58
20-30	1.99	1.25	62
30-50	3.85	2.45	61

Both of the enzyme activities were thus found in all the alcohol precipitates. The highest activity was found in the fraction precipitated by 30-50% alcohol.

If, however, we compare the ratio of the amount of reducing sugars produced from dextran to that from glycogen, expressed as mg of glucose per ml, we find that it has a practically constant

TABLE 4

Effect of Inhibitors on the Activity of α-1,4- and α-1,6-Polyglucosidases

Duration of incubation, hr	Glycogen		Dextran	
	control	experiment	control	experiment
p-Chloromercuribenzoate ($5 \cdot 10^{-5}$ M)				
3	0,640	0,350	0,030	0,016
24	1,100	0,790	0,090	0,020
3	—	—	0,500*	0,230*
Histidine hydrochloride (0.1 M)				
3	0,660	0,700	0,060	0,070
24	1,680	1,710	0,750	0,730

*Prepared from beef liver.

value of about 60%. Fractional precipitation with alcohol does not, therefore, lead to any separation of enzymes hydrolyzing glycogen and dextran.

Separation of the proteins of the enzyme preparation by starch slab electrophoresis. The histograms of Fig. 1 illustrate the results of electrophoretic fractionation of the enzyme proteins, together with the hydrolytic activity of the fractions with respect to glycogen and dextran. Electrophoresis was conducted at pH 4.8 (Fig. 1A) and at pH 7.8 (Fig. 1B).

Our results showed that the enzyme preparations contained very little ballast protein, devoid of enzymatic activity; the rate of hydrolysis of glycogen and dextran varied for the different fractions, but the ratio of the activities remained constant. Starch slab electrophoresis at different pH values did not, therefore, lead to any separation of enzymatic activities.

Ion exchange chromatography of enzyme preparations. Fractional elution from a carboxymethylcellulose column of the proteins present in an enzyme preparation gave three small protein peaks, with no enzymatic activity, and one large peak, in which all of the enzyme activity was concentrated in a few fractions only (Fig. 2). However, in this fraction, too, hydrolysis of glycogen was always associated with hydrolysis of dextran.

Comparative Study of the Properties of α-1,4- and α-1,6-Polyglucosidase

Effects of ageing and of incubation at various pH on dextranolytic and glycogenolytic activities. A portion of the enzyme solution was stored for several days in the cold, at pH 4.8, a second portion at pH 7.0 (neutralized with dry sodium carbonate), and a third at pH 3.5.

The solutions were then incubated with glycogen and dextran at pH 4.8. We found that the activity of the preparation fell by 40-50% after 2-3 days of storage at pH 7.0 (Table 1). Storage at pH 3.5 led to total loss of activity. We could discern no differences in the relative rates of hydrolysis of dextran and glucose by fresh and aged preparations. The activities with respect to both substrates fell parallel in all cases. The same applied to preparations stored at pH 4.8, and incubated with dextran and glycogen at pH 7.0 (Table 2). Decomposition of dextran and of glycogen is thus affected to the same extent by previous storage of the enzyme solutions at different pH, and by subsequent incubation with the substrates at different pH.

Effect of sugars on the enzymatic hydrolysis of dextran and glycogen. It has been shown [7] that isomaltose and glucose have an inhibitory effect on dextranglucosidase. Our experiments show that these sugars also inhibit hydrolysis of glycogen by our enzyme preparations (Table 3).

Effects of p-chloromercuribenzoate and of histidine on the activity of the enzyme preparation. At a concentration of $5 \cdot 10^{-5}$ M, p-chloromercuribenzoate causes a 30-40% inhibition of hydrolysis of dextran and glycogen (Table 4). Histidine has no effect on the hydrolysis of these substrates. Thus, addition of histidine or of

TABLE 5

Comparison of the Activities of Various Preparations of Liver Enzyme. Increase in reducing power, expressed as mg of glucose per ml

Date of preparation	Incubated for 3 hr			Incubated for 24 hr		
	glycogen	dextran	dextran/glycogen %	glycogen	dextran	dextran/glycogen %
11/27/58	0,925	0,615	65	—	—	—
12/30/58	0,615	0,385	62	—	—	—
1/25/58	0,615	0,465	65	—	—	—
3/27/58	0,900	0	—	1,16	0,10	9
6/10/59	0,280	0	—	0,83	0,08	10

p-chloromercuribenzoate to the enzyme systems did not reveal any differences in the enzymatic decomposition of glycogen and dextran, or provide any evidence of separation of the two enzyme activities. The effect of these inhibitors on different carbohydrases is highly specific. Thus, α-1,6-amyloglucosidase, which catalyzes the hydrolysis of α-1,6-linkages at branching points of the glycogen molecule, is totally inhibited by p-chloromercuribenzoate, but not by histidine [11]. On the other hand, α-1,6-oligoglucosidase, which attacks isomaltose and low molecular weight saccharides, is inhibited by histidine, but not by p-chloromercuribenzoate.

Both of the activities fell to an equal extent when the preparations were heated in acid solution at over 55°. Sodium chloride had no activating effect on the enzyme systems, with either substrate.

Seasonal differences in the activity of α-1,4- and α-1,6-polyglucosidases. Observations extending over a number of years showed that liver (but not spleen) dextranglucosidase activity was scarcely detectable during the spring and summer months.

We found that although "spring" preparations of dextranglucosidase had practically no effect on dextran, they were quite active with respect to glycogen. The data of Table 5 show that when glycogen was incubated for 3 hr with enzyme from the liver of an animal killed on March 27th, the increase in the reducing power of the system, expressed as mg of glucose per ml, amounted to 0.9, which is much the same as was found with enzyme preparations of December 30th, November 27th and January 25th; this preparation had, however, no effect on dextran. When dextran was treated with "winter" enzyme preparations, however, the increase in reducing power varied from 0.4 to 0.6, and the dextran/glycogen ratio was 60-65% (compare with the data of Tables 1, 2 and 3).

Incubation of "spring" preparations of enzyme with glycogen for 24 hr gave an increase in reducing power equivalent to about 1 mg of glucose per ml, as compared with about 0.1 mg/ml for incubation with dextran; the dextran/glycogen ratio was about 10%, viz., between a sixth and a seventh of the winter ratio.

DISCUSSION OF RESULTS

Our experiments showed that preparations of dextranglucosidase from the liver and spleen, attacking terminal α-1,6-linkages of dextran, are also active with respect to terminal α-1,4-linkages of glycogen, giving rise to glucose and to high-molecular dextrins giving a brownish-yellow color with iodine, and resembling the β-dextrins in a number of respects. The linkages at the branching points of the two substrates are not attacked. Miller and Copeland's blood enzyme differs from our preparations in having a different pH optimum, and in not attacking dextran. It is not possible to state with certainty whether α-1,4-polyglucosidase is a separate enzyme, resembling Miller and Copeland's blood enzyme or the γ-amylases of molds, or whether dextranglucosidase does not possess rigorous specificity, but is able to attack both α-1,6- and α-1,4-linkages of different polyglucosides; the two activities are very closely associated. They could not be separated by means of fractional precipitation with alcohol, by starch slab electrophoresis under different conditions, or by ion exchange chromatography. Nor could any evidence of separation be derived from a study of the activities of the enzymes in the presence of various inhibitors, or at different pH values and temperatures. Only on the basis of observed seasonal variations in the acitivity of the enzyme preparations was it possible to conclude that they contain two separate

polyglucosidases, since the almost complete disappearance of dextranglucosidase activity from rabbit liver during the spring and summer months was not associated with any substantial fall in α-1,4-polyglucosidase activity, so that considerable degradation of glycogen could be achieved by preparations which did not attack dextran (α-amylase was absent from the preparations).

It is known that when rabbit liver glycogen is treated with α-amylase polyglucosides are formed, which differ from glycogens and dextrans in possessing α-1,4- and α-1,6-linkages in equal numbers [12]. It is conceivable that the breakdown of these polyglucosides is achieved by the synchronous action of the two polyglucosidases (α-1,6-dextranglucosidase and α-1,4-polyglucosidase), to which may be ascribed the close association of the two enzymes, and the close similarity of their properties.

Work is now in progress on the elucidation of this question.

SUMMARY

Enzyme preparations prepared from the liver and spleen possessed α-1,4- and α-1,6-polyglucosidase activities. These preparations, which did not possess any α-amylase activity, detached terminal glucose residues from glycogen and dextran molecules. Separation of the two activities could not be achieved by means of fractional precipitation with alcohol, by starch slab electrophoresis at different pH, or by ion exchange column chromatography.

Glucose and isomaltose cause equal inhibition of enzymatic hydrolysis of glycogen and dextran. Histidine has no effect on either activity, and p-chloromercuribenzoate inhibits both to an equal degree. Preliminary storage of the enzyme preparations at pH 7 lowers both activities to the same extent.

The optimum pH for the activity of the enzyme preparation is 4.8, with both dextran and glycogen. Only very slight hydrolysis of dextran and glycogen is found at pH 7. α-1,6-Polyglucosidase activity of rabbit liver is practically absent during the spring and summer months, whereas the α-1,4-polyglucosidase activity is retained.

LITERATURE CITED

[1] Y. Tsujiskaka, J. Fukumoto and T. Yamomoto, Nature 18, 770 (1958).

[2] D. Goulston, Austral, J. Sci. 10, 85 (1947).

[3] K. Miller and W. Copeland, Biochim. et biophys. acta 22, 193 (1956).

[4] E. L. Rozenfel'd, Biokhimiya 21, 84 (1956).

[5] E. L. Rozenfel'd and I. S. Lukomskaya, Clin. Chim. Acta 2, 105 (1957).

[6] E. L. Rozenfel'd, Doklady Akad. Nauk SSSR 128, 6 (1959).*

[7] I. S. Lukomskaya and E. L. Rozenfel'd, Biokhimiya 24, 261 (1958).

[8] O. Lowry, J. Rosenbrongh, L. Farr and R. Randall, J. Biol. Chem. 193, 265 (1951).

[9] E. Bacon, and J. Bacon, Biochem. J. 758, 396 (1954).

[10] E. V. Goryachenkova, "Method of Separation of Proteins, Using Cellulose-Based Ionites." Methodological Letter, Trudy Inst. Biol. i Med. Khim. AMN SSR, Moscow (1959).

[11] J. Larner and R. Gillespie, J. Biol. Chem. 223, 709 (1956).

[12] E. L. Rozenfel'd, Biokhimiya 24, 879 (1958).

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PHOSPHORUS COMPOUNDS IN DEVELOPING CULTURES OF AZOTOBACTER VINELANDII

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A previous communication from this laboratory dealt with the phosphorus compounds in Az. agile during the life-cycle of the cell. Particular attention in that study was paid to the polyphosphates, which are a very important functional cell component, supplying both phosphorus and energy for the cell's synthetic processes.

The present work deals in greater detail with the phosphorus compounds of Az. vinelandii and with the quantitative changes in these compounds in the process of development.

It is not excluded that the polyphosphates, known to be energy donors and stores in many lower animals, are also energetically concerned in nitrogen fixation [2]. In the present work this possibility was tested by comparing the quantitative changes in the various phosphorus compounds, and particularly in polyphosphates, in nitrogen-fixing cultures of Az. vinelandii, with those of a similar culture in which nitrogen fixation has been suppressed by growing in the presence of ammonium salts. At the same time attempts were made to study closer the possible mechanisms of polyphosphate synthesis, and also their utilization by the cell.

MATERIALS AND METHODS

The Az. vinelandii cultures used (obtained from the Museum of the Microbiology Dept., Moscow State University) were grown on the nitrogen-free medium of Bourke, or on the same medium with ammonium salt supplementation. The conditions of culture and growth characteristics of Az. vinelandii were very similar to those described previously [3] for Az. agile (there was only a slight difference in the growth pattern of the first culture). Az. vinelandii cultures were harvested at the lag, logarithmic, and stationary phases for the study of their phosphorus metabolism; two-day old inoculating stocks were also used.

In nitrogen-fixing cultures the lag phase occupied the first 10-15 hours after inoculation, the logarithmic phase – from the 10-15 hour to the 30th hour of incubation and the stationary phase – from the 30th hour onwards. Az. vinelandii cultures grown in the presence of ammonia-nitrogen remained in the lag phase in the first 8-12 hours of incubation, in the logarithmic phase from 8-12 to 30 hours, and in the stationary phase from the 30th to 45th hour of incubation.

The freshly harvested bacterial mass was fixed by freezing at low temperatures. Treatment with ethanol and ether for fat extraction was omitted, since it was observed that a certain loss of acid soluble, phosphorus-containing compounds (phosphorylated carbohydrates and particularly mononucleotides) could occur through extraction into ethanol.

The Acid Soluble Fraction. The bacterial pellet was extracted twice for 30 min in the cold with 5% TCA, then with 2% TCA and with water. The extracts and washings were pooled, brought to a known volume, and samples of the combined extract were taken for the estimation of various acid-soluble phosphorus fractions: orthophosphate (directly, P_i), phosphate released after 7- and 30-min hydrolysis in N HCl at 100° (P_7 and P_{30},

respectively) and P_{Tot} of the TCA extract (after wet-ashing with $HClO_4$). From these data were calculated values for P_{23} ($P_{23} = P_{30} - P_7$). This value corresponds to the acid-soluble polyphosphates [4]. This calculation allows the correction for the phosphorus formed as a result of the partial hydrolysis of fructosediphosphate, ATP, ADP and other acid-soluble labile phosphates. The total labile phosphate of the extract was calculated as the difference between P_7 and P_i, and the stable acid-soluble phosphorus as the difference between P_T and P_7. All phosphorus determinations were carried out by the method of Berenblum and Chain, as modified by Weil-Malherbe and Green [5].

Apart from the general fractionation of the groups of acid-soluble compounds, selective specific fractionation was also carried out. Acid-soluble nucleotides were adsorbed on Norit. The samples of TCA extracts were shaken with two portions of Norit in succession for 30-40 min in the cold (100 mg of Norit was used for each volume of extract representing 1 g of wet weight of bacteria). According to published results [6], inorganic phosphate and sugar esters are not adsorbed in this procedure. Control experiments showed that the acid-soluble pholyphosphates also remained unadsorbed by Norit under these conditions. The charcoal containing the adsorbed nucleotides was centrifuged off and washed several times with water. The nucleotides were eluted from the charcoal with five successive (low volume) washes of 45% ethanol containing 1% ammonia [7]. The completeness of adsorption and elution was checked by estimating the extinction of the solutions at 267.5 and 290 mμ, using a SF-4 spectrophotometer. If the extinction of the TCA extract was taken as 100%, the combined extinctions of the eluates rarely exceeded 85%. In subsequent work, therefore, nucleotides in the extract were determined by the difference in P_7 before and after Norit treatment. This allowed a rapid estimation of the total and labile nucleotide-P content of the extracts.

Direct precipitation of the nucleotides from the TCA extracts as mercury salts (without the preliminary adsorption and desorption from charcoal) was found to be unsatisfactory.

On some occasions the eluates from the charcoal contained, apart from the nucleotides, also a bacterial polysaccharide which was present in the TCA extracts of Azotobacter. This was removed by treating the eluates with two volumes of 96% ethanol. The precipitate (containing no phosphorus) was washed with dilute ethanol and the nucleotides in the combined supernate and washings were precipitated from the solution as the barium salts (according to Umbreit) at pH 7.

The Ba salts of the nucleotides were solubilized by shaking the aqueous suspension with a small amount of the cation exchanger KU-2 (X4, in the H^+ form) to the complete solution of the precipitate* or by the precipitation of the Ba as $BaSO_4$ with 0.1 N H_2SO_4. Labile and total P, and also ribose (by the orcinol reaction [8]) were estimated in the final solution.

After removal of the nucleotide fraction the remainder of the TCA extract was filtered through a fine asbestos filter in order to remove fine charcoal particles which were not brought down by centrifugation. Solid $Ba(OH)_2$ was added to bring the pH of the solution to 4.0-4.5. Barium acetate was then added, the solution allowed to stand overnight and the barium salts of the soluble polyphosphates which precipitated out were collected, washed carefully with water and rendered barium-free as described above. Orthophosphate, P_7 and total P were estimated in this solution. This fraction was found to consist entirely of acid soluble polyphosphates, since the entire phosphorus was mineralized in 7 min.

Solid $Ba(OH)_2$ was again added to the supernatant from the polyphosphate precipitate, until the pH was 8.2 (to phenolphthalein), followed by four volumes of ethanol. The precipitate which separated in the cold, and which contained the Ba-salts of various P esters and of orthophosphate, was collected and solubilized as described above. Orthophosphate, P_7 and total P were also determined in this fraction. Other assays on this solution included total reducing power with anthrone [9], pentoses with orcinol [8] and fructose according to Kulka [10].

The Lipid Fraction. The lipids were removed from the TCA-extracted bacterial pellet by repeated extraction at room temperature with ethanol-ether mixtures (3:1) for 2-3 days. Total phosphorus in the lipid fraction was estimated after wet-ashing. Lipid extraction with hot solvents was avoided in order to prevent possible depolymerization of polyphosphates.

The Acid-Insoluble Fraction. The acid-insoluble phosphorus compounds were fractionated into the DNA and RNA fractions by a somewhat modified procedure of Schmidt and Thannhauser [11].

*In some cases the mixture of free nucleotides was fractionated by means of ion-exchange chromatography into single components.

TABLE 1

Phosphorus Compounds in the RNA Fraction of Az. vinelandii in μg/10^9 Cells

Age of culture, h	RNA-P, spectrophotometric assay at 260 mμ		RNA-P determined by formula: $\frac{E_{270}-E_{290}}{0,19}$		RNA-P estimated as ribose		RNA-P estimated as purine-N	RNA-P estimated as the sum of nucleotides separated electrophoretically
	I	II	I	II	I	II	II	II
Inoculating stock	7,41	8,28	7,40	7,84	5,07	10,08	6,34	6,05
4	—	12,84	—	10,56	—	12,32	9,83	9,70
5	9,26	—	6,66	—	6,52	—	—	—
8	—	20,90	—	16,65	—	15,97	12,71	12,51
10	11,90	—	8,40	—	7,90	—	—	—
12	—	20,25	—	19,26	—	18,90	15,07	14,73
15	11,71	—	8,27	—	8,15	—	—	—
18	—	11,37	—	9,23	—	10,55	8,42	8,50
24	9,53	10,64	7,64	9,23	7,03	10,61	8,41	8,22
30	—	9,94	—	8,18	—	10,11	8,07	8,00
36	8,03	—	6,56	—	6,25	—	—	—
42	—	8,71	—	7,77	—	8,19	6,70	6,97
48	7,64	—	6,58	—	5,58	—	—	—

Age of culture, h	P of UV-absorbing compounds, other than RNA (excess P of RNA fraction)		P of UV-non-absorbent compounds		Total RNA-P		Insoluble polyphosphate-P	
	II	II	I	II	I	II	I	II
Inoculating stock	2,34	2,23	2,62	2,43	10,00	14,71	2,31	4,00
4	—	3,14	—	1,96	—	18,76	—	3,96
5	2,74	—	1,55	—	13,81	—	3,00	—
8	—	7,74	—	0,16	—	25,06	—	4,65
10	4,00	—	0,23	—	15,43	—	3,30	—
12	—	5,52	—	0	—	24,55	—	4,30
15	3,56	—	0	—	12,30	—	3,59	—
18	—	2,87	—	1,97	—	15,34	—	2,00
24	2,50	2,42	1,93	2,27	13,02	16,75	1,56	3,04
30	—	1,94	—	2,30	—	17,46	—	5,22
36	1,78	—	2,15	—	12,06	—	1,88	—
42	—	1,64	—	2,47	—	15,42	—	4,34
48	2,06	—	2,60	—	10,10	—	2,47	—

Note: I) nitrogen-fixing culture; II) culture grown with ammonium salts. The inorganic ("Delory") P in the RNA fraction in all cases was either completely absent, or present in negligible trace amounts, which indicated the absence of phosphoproteins in Azotobacter.

The RNA Fraction. Assays on this fraction comprised orthophosphate, total phosphorus, P_7, and P_{30}. The phosphorus of the insoluble polyphosphates is generally given as the difference between P_{30} and P_7 [4]. It should be noted that this calculation was not applied in the present work since in the case of Azotobacter it frequently led to a negative value. The phosphorus of the insoluble polyphosphates was therefore calculated as the P_7, corrected for mineralization of nucleotide P. Separate tests on the value of this correction showed that 7 min hydrolysis in 1 N HCl at 100° mineralized about 23-25% of the RNA in azotobacter – a value in agreement with that obtained by Ebel [12] for yeast RNA.

RNA-P was estimated by calculation from spectrophotometric determination of optical densities at 270 and 290 mμ [13] and also at 260 mμ [14], assuming E_p = 10,000. The method of spectrophotometric estimation, however, was found to be unsatisfactory in Azotobacter, since absorption at 260 mμ in the material at hand was

TABLE 2

Phosphorus Content of the DNA, Bacterial Residue and Phospholipid Fraction in Az. vinelandii in μg/10^9 Cells

Age of culture, h	DNA-P determined spectrophotometrically		Excess-P in DNA fraction		P-total in DNA		Bacterial residue-P		Phospholipid-P	
	I	II	I	II	I	II	I	II	I	II
Inoculating stock	1,15	1,37	4,12	4,01	5,27	5,38	1,10	2,11	3,60	2,67
4	—	1,89	—	6,38	—	8,27	—	2,38	—	2,96
5	1,28	—	4,70	—	5,98	—	0,84	—	3,41	—
8	—	2,20	—	6,74	—	8,94	—	0,27	—	3,24
10	1,60	—	2,61	—	4,21	—	0,48	—	3,20	—
12	—	1,44	—	9,12	—	10,56	—	2,29	—	5,04
15	1,53	—	3,10	—	4,63	—	1,95	—	5,14	—
18	—	1,49	—	3,46	—	4,95	—	2,91	—	2,50
24	1,14	1,48	1,40	4,10	2,54	5,58	1,35	3,66	4,19	2,98
30	—	1,39	—	5,30	—	6,69	—	3,84	—	3,21
36	1,13	—	2,00	—	3,13	—	1,08	—	3,46	—
42	—	1,28	—	6,11	—	7,39	—	2,20	—	2,72
48	1,18	—	2,71	—	3,89	—	0,90	—	3,16	—

Note: I) nitrogen-fixing culture; II) culture grown with ammonia-N.

not confined to RNA alone and was produced also by other P-compounds present. The value of E_p for RNA also varied in different bacterial species [15].

RNA determination by the ribose method with orcinol was quite satisfactory for the nitrogen-fixing cultures of Az. vinelandii, but in the case of the bacteria grown in the presence of ammonium salts excessively high values were produced due to the presence in these cultures of a pentose-containing polysaccharide [16]. RNA determination through the estimation of purine-N was reliable, but required a large amount of material [3].

The most accurate method for the determination of RNA involved the electrophoretic separation of the ribonucleotides on paper. Electrophoresis was carried out on washed "Leningrad Fast" paper, in 0.075 ammonium acetate buffer pH 3.5, with a potential of 540 v over 5-6 hours [17]. The quantitative calculations were carried out using the coefficients proposed by Elson et al [18]. At the completion of the run, UV-absorbing nonnucleotide material either remained on the origin, or traveled a short distance towards the cathode, producing two distinct absorbent bands, clearly visible when the paper was examined under a UV light.

Treatment of electrophorograms with a phosphorus-developing reagent [19] revealed the presence in the RNA fraction of some nonUV-absorbent, phosphorus-containing compounds. It is possible that these compounds are analogous with the phosphate esters described by Mitchell and Moyle [20]. In a recent communication of Stacey et al [15] reference was also made to the presence in the RNA of a number of Gram-positive and Gram-negative bacteria of excess P, comprising some phosphorus-containing compounds which absorb in the UV, but are not RNA nucleotides.

If the total P of the RNA fraction is designated a; RNA-P, determined by purine-N analysis or by the nucleotide-electrophoretic method, — b; insoluble polyphosphate P — c; RNA-P, determined by spectrophotometry of the whole fraction at 260 mμ — d; then the excess P (total) of the RNA fraction (A) = a — (b + c); the excess P of the UV-absorbent, nonnucleotide P-compounds (B) = d — b; the excess P of the RNA fraction of UV-nonabsorbent compounds (C) = B — A.

The values for RNA-P in Azotobacter, determined by various methods, and also the values for the excess P of UV-absorbent and nonabsorbent compounds, are shown in Table 1.

The DNA Fraction. The DNA component of the precipitate obtained after the acidification of the alkaline extract of the acid insoluble, fat-free fraction, was obtained by two consecutive extractions of 30 min each with 0.5 N $HClO_4$ at 100°. Total P and DNA-P (spectrophotometrically — [13]) were determined in this fraction. Phosphorus analysis of the DNA fraction revealed the presence of a large amount of excess phosphorus, at levels greatly exceeding those of DNA-P itself.

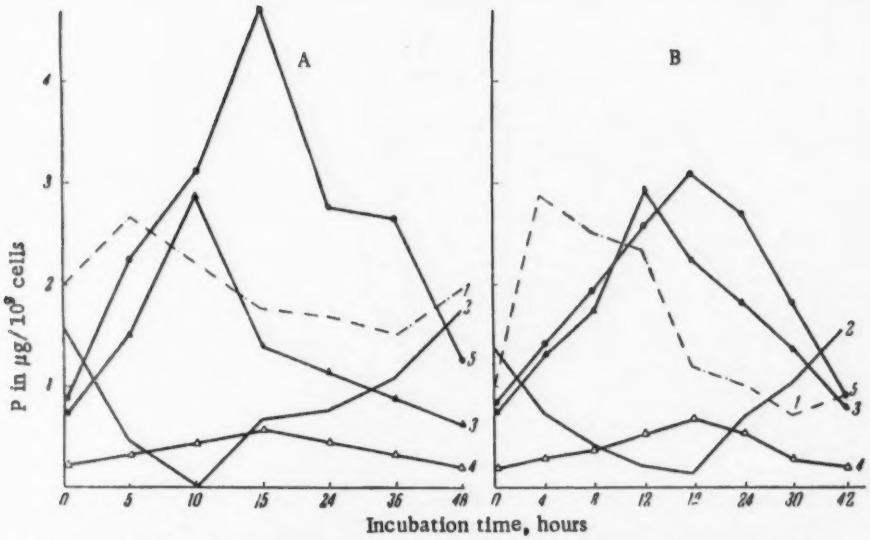


Fig. 1. The change in acid-soluble phosphorus content during the incubation of Az. vinelandii: A) nitrogen-fixing culture; B) culture receiving ammonia-N: 1) orthophosphate; 2) acid-soluble polyphosphates; 3) total nucleotide phosphorus; 4) labile nucleotide phosphorus ("The ATP-ADP system"); 5) total phosphorus of sugar esters.

TABLE 3

Total-N, Protein-N, RNA-P, Insoluble Polyphosphate-P and Mononucleotide-P in the Cell Mass of Az. vinelandii Contained in 1 ml of Culture Medium

Age of culture, h	Total N		Protein-N		RNA-P		Insoluble polyphosphate-P		Labile nucleotide P (ADP-ATP)		Polyphosphate-P/Total-P	
	I	II	I	II	I	II	I	II	I	II	I	II
Inoculation stock	21,75	20,44	23,13	17,22	0,91	1,09	0,42	0,72	0,04	0,03	1,93	3,52
4	—	21,22	—	16,44	—	1,55	—	0,63	—	0,05	—	2,96
5	22,22	—	18,77	—	1,04	—	0,48	—	0,06	—	2,16	—
8	—	28,88	—	21,68	—	2,13	—	0,79	—	0,06	—	2,73
10	20,65	—	17,03	—	1,26	—	0,53	—	0,08	—	2,56	—
12	—	130,37	—	100,40	—	9,57	—	2,80	—	0,33	—	2,15
15	71,50	—	60,23	—	4,08	—	1,80	—	0,30	—	2,51	—
18	—	336,6	—	290,95	—	19,12	—	4,50	—	1,40	—	1,34
24	154,70	344,3	133,91	296,11	7,87	20,22	1,75	7,48	0,58	1,35	1,13	2,17
30	—	337,1	—	288,00	—	21,60	—	14,09	—	0,76	—	4,17
36	187,40	—	162,85	—	9,50	—	2,86	—	0,47	—	1,52	—
42	—	341,1	—	289,60	—	22,30	—	13,00	—	0,64	—	4,07
48	222,30	—	197,27	—	9,49	—	4,20	—	0,39	—	1,89	—

Note: I) nitrogen-fixing culture; II) culture grown on ammonia-N.

Bacterial residue. The residue remaining after the removal of acid-soluble-P, lipids, RNA and DNA, was dissolved in alkali. Total P was determined on this solution.

The phosphorus content of the DNA fraction, excess P, bacterial residue and phospholipid are shown in Table 2.

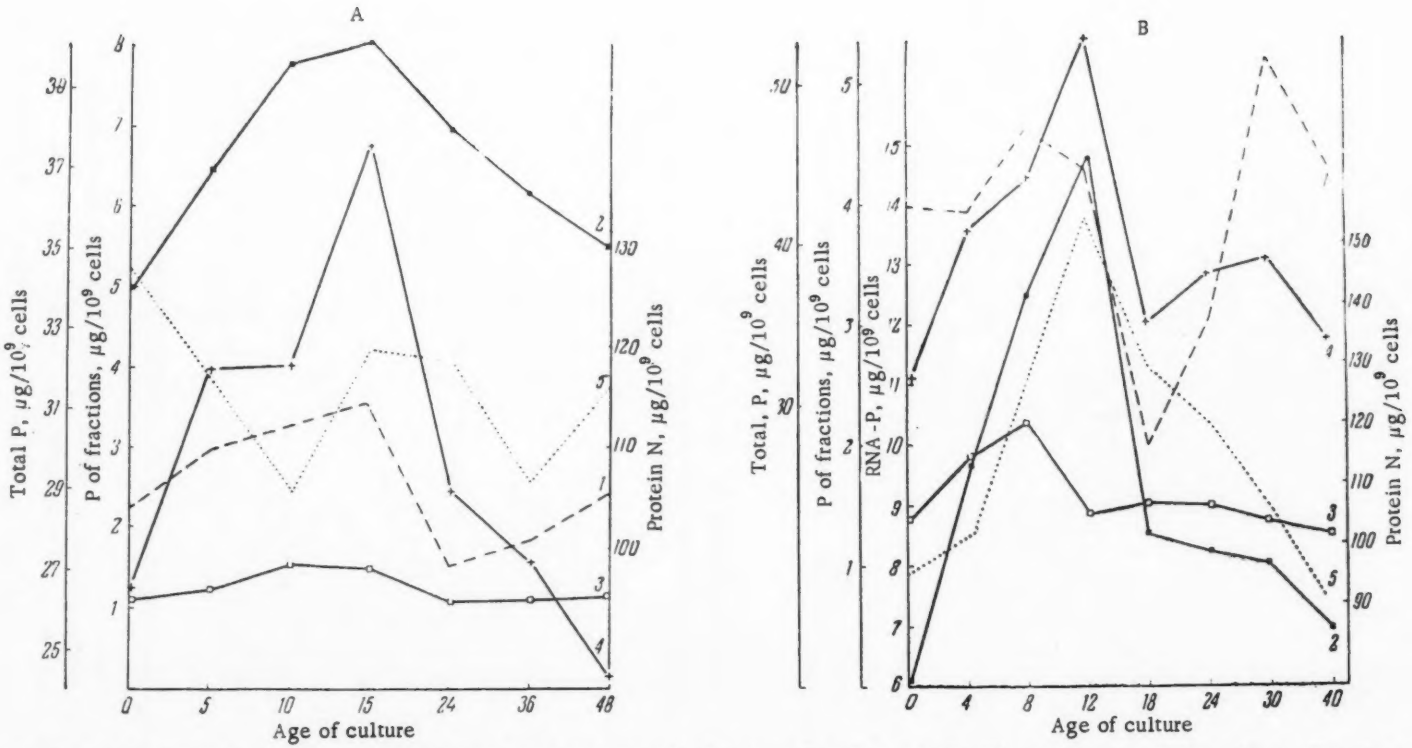


Fig. 2. The change in the phosphorylated sugars content during the development of Az. vinelandii: A) nitrogen-fixing culture; B) culture grown with ammonia nitrogen:

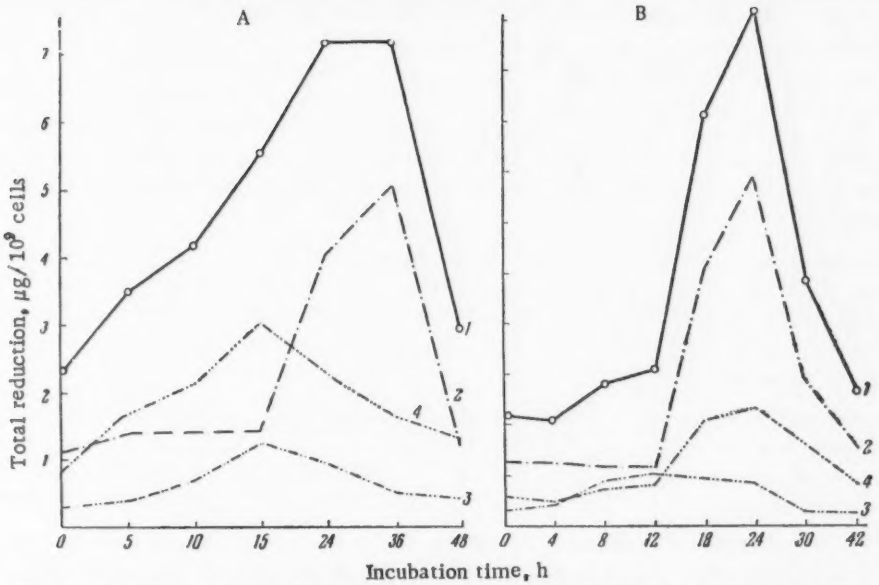


Fig. 3. The change in the phosphorylated sugars content during the development of Az. vinelandii: A) nitrogen-fixing culture; B) culture grown with ammonia nitrogen: 1) total sugar ester content (as glucose); 2) glucose phosphates; 3) fructose phosphates; 4) pentose phosphates.

RESULTS

Azotobacter in the latent phase took up orthophosphate from the medium in large amounts (Fig. 1), thus creating favorable intracellular condition for the synthesis of organic phosphorus compounds. With the growth and multiplication of the culture the orthophosphate content per cell fell considerably. This was not due to the decreased rate of orthophosphate uptake from the medium, but was caused by the distribution of the available phosphate between a continuously increasing number of new cells and also by the passage of the intracellular phosphate from the inorganic phase into the various organic compounds. Indeed, in the period of maximal growth (logarithmic phase) the polyphosphate content per unit bacterial mass increased considerably (cf. Table 3).

Calculation on the cell-number basis showed without doubt that intensive accumulation of the acid-insoluble polyphosphates and nucleic acids, particularly RNA, took place during the latent phase (Fig. 2). During that phase orthophosphate was the prime source of phosphorus for the synthesis of the insoluble polyphosphates and nucleic acids. This follows from the observation that, in the second part of the latent phase, during the intensification of polyphosphate and RNA synthesis, cellular orthophosphate became considerably depleted (Figs. 1 and 2).

It appeared from the experimental results obtained that the synthesis of polyphosphates and nucleic acids, although proceeding at the expense of orthophosphate, did not take place directly from this precursor, but indirectly, through the mediation of certain acid-soluble phosphorus compounds, the dynamics and turnover of which would be such as to correspond with the rate of polyphosphate accumulation and nucleic acid synthesis during growth (Figs. 1 and 2). Furthermore, amongst the acid-soluble organic phosphate esters, the sugar phosphates, and particularly fructose and pentose phosphates, showed good correspondence with the accumulation of the insoluble polyphosphates and RNA, which suggests that the esters of this group, through their interaction with the phosphorus of the "ATP-ADP system"*, may participate in the synthesis of the polyphosphates and nucleic acids (Figs. 1, 2A and 3). The facts established in the present work are in agreement with some published data [21-25].

*The phosphorus of ATP and ADP accounts for 60-70% of the labile P of the soluble nucleotides in Az. vinelandii in the stationary phase, and the specific activity of those nucleotides exceeds the specific activities of other nucleoside di- and triphosphates. On this basis the labile phosphorus of the acid-soluble fraction was conditionally designated as the P of the "ATP-ADP system."

It will be seen from Fig. 1 that the peak values of mononucleotide accumulation did not occur at the same time from the onset of incubation in the cultures grown on molecular nitrogen and in those where the source of nitrogen was ammonia. Thus, in the nitrogen-fixing culture the maximum level of nucleotide content occurred during the second half of the lag phase (10 h), while in the culture supplied with ammonia it occurred at the end of this phase (12 h). As was shown elsewhere [3], in cultures grown on the nitrogen-free medium of Bourke, the level of total nitrogen in the cell remained constant throughout the lag phase. Consequently in such a culture mononucleotide synthesis can only proceed at the expense of amino acids derived from protein breakdown in the early lag phase (cf. Fig. 2A).

The sharp fall in nucleotide content in the nitrogen-fixing culture between the 10th and 15th hour of incubation was apparently due to the continuous synthesis of nucleic acids, particularly RNA, during that stage, and following a lag phase during which nitrogen fixation was negligible (Figs. 1A and 2A). Apart from this, after the 10th hour of incubation protein breakdown in the cells of nitrogen-fixing Azotobacter ceases, and their intensive synthesis commenced (Fig. 2A).

In cultures grown in the presence of a nitrogen source the continuous increase in nucleotide content up to the end of the lag phase (Fig. 1B), concomitant with vigorous synthesis of RNA (Fig. 2B) can be explained by the fact that here the nucleotides were mainly synthesized not at the expense of protein breakdown products, but from the supplied medium nitrogen.

The somewhat retarded rate of increase in nucleotide content in the cultures where nitrogen-fixation had been suppressed, compared with the nitrogen-fixing cultures (Fig. 1), was apparently due to the more intensive utilization of the nucleotides for RNA synthesis in the nitrogen-supplemented cultures, where RNA content in the latent phase was about 1.5 times that in the nitrogen fixing culture (Fig. 2). It is characteristic that in the logarithmic phase the mononucleotide content of the ammonia nitrogen supplied culture was considerably higher than in the culture using molecular nitrogen (Fig. 1). This indicates the more intensive biosynthetic activity in the former culture. It was of interest to note that the dynamics of nucleotide synthesis in the development of Az. vinelandii cultures, both using molecular and ammonia-nitrogen, were very similar to that of Az. agile cultures [26].

It was also found that the fall in pentose phosphates in the developing azotobacter was concomitant with the increase in nucleotide content (Figs. 1 and 3). This was particularly noticeable in cultures supplied with ammonia nitrogen, where the rates of RNA synthesis were high. This observation may be taken as further proof of the interrelationship between pentose phosphates and those nucleotides involved in nucleic acid synthesis [27].

It is usually believed that polyphosphates exist in two forms: the relatively low molecular weight compounds (acid-soluble polyphosphates) and the high molecular weight polymers (acid-insoluble). The latter apparently exist in the cell in bound form and have a high degree of physiological activity. According to results obtained in this laboratory [28-31], the insoluble polyphosphates become activated by binding to nucleic acids. The inoculating stock cultures usually contained high amounts of soluble polyphosphates (Fig. 1). These soluble forms disappeared during the lag phase and this was accompanied by an increase in the insoluble polyphosphates. Two pathways for the disappearance of the soluble polyphosphates may be considered: they could be depolymerized, or else they may be directly polymerized to higher molecular weight forms. The occurrence of both processes appears possible from the study of Figs. 1 and 2. Indeed, the coincidence in the lag phase of the disappearance of the soluble polyphosphates with the appearance of a certain amount of orthophosphate is not accidental. On the other hand, the concomitant appearance of the insoluble polyphosphates during the same period also suggests the possibility of direct transition of the soluble polyphosphate into the insoluble form. The polyphosphates synthesized in the second half of the lag phase are almost entirely of the insoluble type (Fig. 2). This may be taken as an indication that the primary polyphosphate synthesis takes place in the insoluble fraction [21, 30-33]. With the onset of cell division (beginning of the logarithmic phase) the insoluble polyphosphate content per cell fell sharply, which was due not only to the distribution of the polymer over a larger number of cells, but also to the utilization of this energy store in synthetic processes. The decline in the rate of cell division in the second half of the logarithmic phase and in the beginning of the stationary phase was also accompanied by a steady increase in insoluble polyphosphate content (Fig. 2).

It is of interest that in the culture grown in the presence of supplied ammonia nitrogen, medium nitrogen became considerably depleted towards the 24th hour and consequently protein and RNA synthesis came to a

standstill (Table 3). This was accompanied by a sharp rise in the insoluble polyphosphate content of the cells between the 24th and 30th hours (Fig. 2B). This indicates the decreased utilization of polyphosphates in the cell whenever synthetic processes became decelerated, allowing the accumulation of the polyphosphates.

In the late stationary phase there occurs an accumulation of the soluble polyphosphates as a result of the depolymerization of the insoluble polymers [30-34]. That this transition takes place in the manner indicated is shown by the following: in Az. vinelandii cultures supplied with ammonia nitrogen, in the 30-42 hour period, when cell division ceased as a result of the depletion of energy sources from the medium, the insoluble polyphosphate content of the cells fell considerably, while the soluble polyphosphate content increased (Figs. 1B and 2B).

A comparison of the quantitative content of the insoluble polyphosphates with the "ATP-ADP system" during the life cycle of the cell (Figs. 1A, 2A and Table 3) suggests that both the synthesis and the breakdown of the polyphosphates occurred through the mediation of the "ATP-ADP system." This conclusion finds confirmation in a number of publications [21, 24, 35-37]. The most intensive utilization of the polyphosphates in synthetic mechanisms coincided with the highest observable levels of "ATP-ADP" in the cell (Fig. 1). Thus, the quantitative levels of the components of this system may be taken as an indication of the intensity of the synthetic processes within the cell. It would appear that the insoluble polyphosphates serve not merely as an energy store for various synthetic activities and as a reserve of phosphate, but can also be used for the regeneration of ATP and, possibly, of other nucleoside polyphosphates [38].

It was observed that, during the lag phase, Az. vinelandii utilized considerably more of the medium energy sources (sucrose and citrate) than at any other phase, when calculated on the cell basis. Apart from this, the energy which is required for the synthesis of high-energy compounds – polyphosphates and nucleic acids – was also liberated at the expense of the breakdown of protein (in the nitrogen-fixing cultures only) and, particularly, of intracellularpolysaccharide of Azotobacter. Thus, in the latent phase, energy accumulation in the form of acid-insoluble polyphosphates and nucleic acids took place at the expense of the oxidation of both endogenous and exogenous resources.

It should be noted that, as a general rule, cell division only commenced when the cell accumulated a sufficient amount of stored energy in the form of acid insoluble polyphosphates and RNA, which also indicates the utilization of these compounds in synthetic processes [39, 40]. Indeed, in the culture grown in the presence of ammonia nitrogen, RNA content in the first 8 hours of the latent phase was doubled, while protein content under the same conditions increased by only 25%. It would thus appear that the RNA accumulation in this phase of development precedes protein synthesis, which commences only after a certain level of RNA in the cell has been reached (Table 3). In the same period (8 hr) the insoluble polyphosphate content increased by only 15%.

In cultures of Az. vinelandii grown in nitrogen-free media, in the first 10 hours of the latent phase RNA content increased by 38%, polyphosphate content – by 30%, while considerable protein breakdown (25%) took place.

A comparison of the two cultures shows that, in the nitrogen-fixing cultures, in the absence of protein synthesis and when the RNA production rate was low, polyphosphate synthesis was considerably higher than in the culture grown with ammonia nitrogen. This may serve as an indication of the utilization of the polyphosphates in the synthesis of protein and RNA in the nitrogen-supplemented culture, where the synthesis rate was comparatively higher.

Calculation on the cell-mass basis revealed intensive synthesis of protein, nucleic acids (particularly RNA), and polyphosphates in the early multiplication stage (Table 3). In Az. vinelandii cultures grown on nitrogen-free media, at the onset of nitrogen-fixation, that is, between the 10th and 24th hour, protein content increased 8-fold, RNA 6-fold and insoluble polyphosphates only 3-fold. In the corresponding interval, in Az. vinelandii cultures grown in the presence of ammonia nitrogen, protein content increased 16-fold, RNA 10-fold, and insoluble polyphosphate – 9-fold.

It follows from Table 3 that: firstly, the rate of protein synthesis was higher than the rate of RNA synthesis, while the latter still exceeded the rate of polyphosphate increase. This was apparently due to the fact that protein synthesis was accompanied by a utilization of both RNA and polyphosphates, but the latter were used at a larger rate than RNA. Secondly, supplementation of Azotobacter culture media with a nitrogen source led to

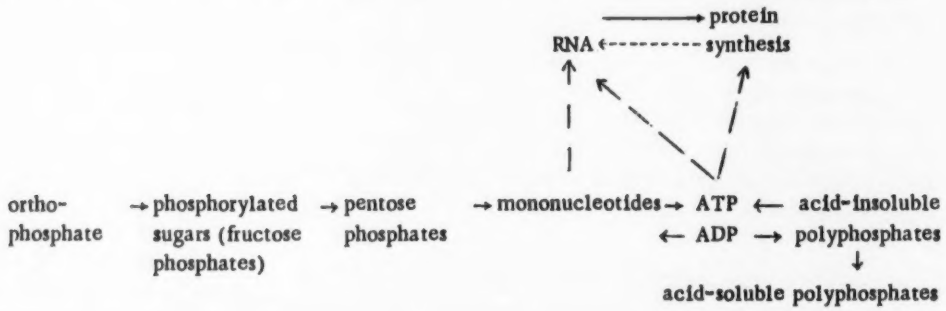
the increased synthesis of not only protein, but also RNA and polyphosphates. Thirdly, during the logarithmic phase (8-24 hour) the rate of increase in polyphosphate content was lower in the culture grown on a nitrogen-free medium than in that supplemented with a nitrogen source. This may serve as an indirect indication that nitrogen fixation is accompanied by an additional demand on the energy resources (possibly polyphosphates). Indeed, in the nitrogen-fixing culture there was less polyphosphate per unit of synthesized nitrogen than in the culture with suppressed nitrogen fixation (Table 3).

It may be suggested that during nitrogen fixation the cell utilizes the energy stored in the polyphosphates, the phosphorus of which is utilized, in the period of maximal nitrogen fixation, for the resynthesis of ATP. The possibility of polyphosphate participation in nitrogen fixation was also suggested by Esposito and Wilson [2].

It is permissible to make another suggestion, namely, that nitrogen fixation is accompanied by the utilization of some precursors (possibly ATP) which also participate in polyphosphate synthesis. As a result of competition for the common precursor and its diversification into other channels during nitrogen fixation, polyphosphate synthesis declines.

The following scheme for the pathways of phosphorus turnover in Azotobacter is suggested on the basis of the present results:

A Scheme for the Pathways of Phosphorus Metabolism in Az. vinelandii



The above scheme provides further detail of the phosphorus metabolism of Azotobacter and corresponds with the basic theories developed in this laboratory [21, 28].

SUMMARY

Phosphorus distribution in various fractions was studied in developing cultures of Az. vinelandii using molecular and ammonia nitrogen. The general growth dynamics of this organism were shown to be similar to those of Az. agile. A comparison of the quantitative distribution of phosphorus in the nitrogen-fixing cultures and in cultures with suppressed nitrogen fixation revealed a considerable phosphorus enrichment of Az. vinelandii cells grown in the presence of a supplemented nitrogen source. This indicated increased rates of phosphorus metabolism in Azotobacter in the presence of ammonia nitrogen.

A number of unidentified phosphorus compounds, both UV-absorbent and nonabsorbent were found in the acid-insoluble fraction of Azotobacter, which usually also contains RNA. The levels of these compounds in the insoluble fraction varied, depending on the time of harvesting.

The neosynthesis of polyphosphates in Azotobacter, as in other organisms containing this group of compounds, took place in the insoluble fraction. Degradation of the high molecular weight polyphosphates, with the concomitant appearance of the low molecular weight forms, took place in the late stationary phase.

The synthesis of nucleic acids and high molecular weight polyphosphates took place with the intermediate formation of fructose phosphates, pentose phosphates, and mononucleotides of the acid-soluble fraction. The formation and utilization of polyphosphates occurred through the mediation of nucleoside di- and triphosphates (the "ADP-ATP system").

The physiologically most active form of polyphosphates is the acid-insoluble form, which fraction can be

utilized in protein and RNA synthesis during certain periods of the cell's life. The polyphosphates should be considered not only as a store of phosphorus and energy, but also as a regenerating system for ATP.

LITERATURE CITED

[1] G. N. Zaitseva and A. N. Belozerskii, Mikrobiologiya 27, 308 (1956).

[2] R. Esposito and P. W. Wilson, Biochim. et biophys. acta 22, 186 (1956).

[3] A. N. Belozerskii, G. N. Zaitseva, L. P. Gavrilova and L. A. Mineeva, Mikrobiologiya 26, 409 (1957).*

[4] K. Lohmann, and P. Langen, Biochim. Z. 328, 1 (1956).

[5] H. Weil-Malherbe and R. H. Green, Biochem. J. 49, 286 (1951).

[6] R. K. Crane, Science 127, 285 (1958).

[7] E. Cabib, L. Leloir and C. Cardini, J. Biol. Chem. 203, 1055 (1953).

[8] G. N. Zaitseva and T. P. Afanas'eva, Biokhimiya 22, 1035 (1957).**

[9] R. Dreywood, Industr. and Engng. Chem. (anal. ed) 18, 499 (1946).

[10] R. G. Kulka, Biochem. J. 63, 542 (1956).

[11] G. N. Zaitseva and A. N. Belozerskii, Mikrobiologiya 26, 722 (1957).*

[12] J. P. Ebel, Bull. Soc. chim. biol. 34, 491 (1952).

[13] A. S. Spirin, Biokhimiya 23, 656 (1958).**

[14] E. Chargaff and S. Zamenhof, J. Biol. Chem. 173, 327 (1948).

[15] A. Jones, S. Rizvi and M. Stacey, J. Gen. Microbiol. 18, 597 (1958).

[16] G. N. Zaitseva, A. N. Belozerskii et al., Mikrobiologiya 28, 58 (1959).*

[17] B. F. Vanyushin and A. N. Belozerskii, Doklady Akad. Nauk SSSR 127, 455 (1959).**

[18] D. Elson, E. Gustafson and E. Chargaff, J. Biol. Chem. 209, 285 (1954).

[19] S. Burrows, F. Grulls and J. S. Harrison, Nature 170, 800 (1952).

[20] P. Mitchell and J. Moyle, Nature 166, 218 (1950); J. Gen. Microbiol. 5, 966, 981 (1951).

[21] E. Bukhovich and A. N. Belozerskii, Biokhimiya 23, 254 (1958);** Doklady Akad. Nauk SSSR 124, 1147 (1959).**

[22] L. Klungsöyr, T. King and V. Cheldelin, J. Biol. Chem. 227, 135 (1957).

[23] M. Szymone, Bull. Acad. polon. sci. Cl II, 5, 379 (1957).

[24] S. Kornberg, Biochim et biophys. acta 26, 294 (1957).

[25] D. Scott and E. Chu, Bacteriol. Proc. 96 (1958).

[26] G. N. Zaitseva, A. N. Belozerskii et al., Mikrobiologiya 28, 675 (1959).*

[27] F. Schlenk, Nucleic Acids (Foreign Lit. Press, Moscow, 1957) p. 250, 275 [Russian translation].

[28] A. N. Belozerskii, Paper to III Internat. Congress of Biochem. Brussels (1955); Paper to IV International Congress of Biochemistry (Vienna, 1958).

[29] V. B. Korchagin, The Metaphosphates of Yeast and the Chemical Nature of Volutin Thesis, M., (1954).

[30] A. N. Belozerskii and I. S. Kulaev, Biokhimiya 22, 29 (1957).**

[31] I. S. Kulaev and A. N. Belozerskii, Biokhimiya 22, 587 (1957);** Doklady Akad. Nauk SSSR 120, 1080 (1958).**

*See English translation.

**Original Russian pagination. See C.B. Translation.

[32] P. Krischnan, S. Damle and V. Bajàj, Arch. Biochem. and Biophys. 67, 1 (1957).

[33] P. Langan and E. Liss, Naturwissenschaften 45, 191 (1958); Biochim. Z. 330, 455 (1958).

[34] L. S. Kulaev, A. N. Belozerskii et al., Biokhimiya 24, 253 (1959).*

[35] A. Komberg, S. R. Kornberg and E. Simms, Biochim. et biophys. acta 20, 215 (1956).

[36] F. Winder and J. Denneny, J. Gen. Microbiol. 15, 1 (1956); 17, 573 (1957).

[37] J. Ebel and G. Dirheimer, Compt. rend. Soc. biol. 151, 979 (1957).

[38] S. Kornberg, J. Lehmann, M. Bessmann, E. Simms and A. Kornberg, J. Biol. Chem. 233, 159 (1958).

[39] S. Mudd, A. Yoshida and M. Koike, J. Bacteriol. 75, 224 (1958).

[30] T. Sall, S. Mudd and A. Takagi, J. Bacteriol. 76, 640 (1958); Arch. Biochem. and Biophys. 60, 130 (1956).

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ELECTROPHORETIC INVESTIGATION OF THE PROTEINS FROM THE GASTRIC MUCOSA OF CATS

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The study of the proteins of the gastric mucosa has up to now been limited almost exclusively to the enzymes of the mucosa, pepsinogen, lipase, urease, and others [1-3]. The first attempt to use the electrophoretic method for the separation of the proteins of the gastric mucosa was made by Demling, Kinzelmeir, and Henning [4]. These authors along with studies of the proteins of other organs carried out a paper electrophoretic separation of gastic mucosa proteins which had been extracted by dilute salt solutions. Cresseri [5] also submitted extracts of pig gastric mucosa to free and paper electrophoresis with the definite problem of studying the chemical and biological properties of the mucoproteins which combined with vitamin B_{12}. We have set ourselves the problem of a more detailed investigation of the composition of the proteins of gastric mucosa in different physiological states in order to explain the relation between secretory and constituent proteins of this tissue.

In the present work we give data on the separation of proteins of gastric mucosa by electrophoresis on agar, which, according to the experiments of a number of authors [6, 7], has a higher separating power than paper electrophoresis.

METHODS

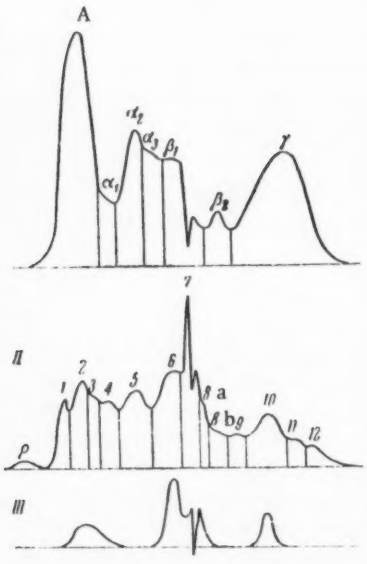
The experiments were carried out on two groups of cats with ten animals in each. The cats of the first group were killed after starvation for 72 hours. The animals of the second group after the same period of starvation were given 30-40 g of meat and after one and a half to two and a half hours from the feeding they were killed by urethane narcosis and exsanguination by passing 1.5-2.0 liters of 0.85% NaCl through the carotid artery. The gastric mucosa without the pyloric part was removed and homogenized in a glass homogenizer with a mixture of equal parts of 2% NaCl and n-butanol (two parts of tissue and one part of mixture) and an extract was prepared from the homogenate by the method of Kaplanskii et al. [8]. The extracts were dialyzed for two hours through a cellophane membrane at 4° against a solution of gum arabic in a borate buffer with pH 8.6 (0.04 M). The resulting extracts contained 1.5-2% protein and were light yellow and slightly opalescent. The extracts were separated by a somewhat modified [14] method of electrophoresis on agar (Grabar [9] and Giri [10]). The thickness of the agar plate was 3 mm; pH of the borate buffer 8.6 and μ 0.04; duration of separation 4.5 hours at a voltage of 2.5 v/cm and current strength 1.6 ma. The electrophoretogram was stained with amido black 10B to show the protein; parallel samples were stained by the method of Uriel and Grabar [11] to detect glucoproteins. The mobility of the resulting fractions was compared with the mobility of protein fractions of cat blood serum obtained by electrophoresis of the serum on agar under the same conditions.

RESULTS

In the extracts of gastric mucosa (from 20 cats in all) we established the presence of at least 13 fractions (figure, curve II). One of the fractions had a considerably greater mobility than serum albumin and it was called the prealbumin fraction, ρ. The remaining fractions were designated by figures from 1 to 12 in accordance with their electrophoretic mobility. Fractions 1, 2 and 3 were not always well separated from each other. In mobility these proteins were close to serum albumin. In some cases the boundary between fraction 1, which is

Relative Content of Protein Fractions of Cat Gastric Mucosa
A) arithemetical average; proteins in % of total amount; K_b) variation coefficient; n) number of animals

Fraction	Starved cats		Fed cats	
	$A \pm \sigma_A$ (n 10)	K_b	$A \pm \sigma_A$ (n 10)	K_b
ρ	0,4		0,5	
1	6,0±0,91	15,2	6,5±0,57	8,8
2	13,5±1,51	11,2	14,6±1,62	11,0
3	7,6±1,17	15,4	6,9±0,77	11,2
4	5,9±0,81	13,7	6,0±1,07	17,8
5	10,7±1,47	13,7	10,0±1,32	13,2
6	13,0±1,02	7,9	11,6±1,19	10,2
7	11,8±1,18	10,4	12,4±1,17	9,4
8	9,5±0,51	5,4	8,7±0,61	7,0
9	6,3±0,49	7,7	6,7±0,41	6,1
10	10,7±0,59	5,5	10,5±0,79	7,5
11	3,4±0,30	8,8	4,3±0,51	11,8
12	1,2±0,14	11,6	1,3±0,14	10,8



Densitometric curves: I) cat blood serum. Stained with amido black 10B; II) soluble proteins of cat gastric mucosa of the same cat. Stained for glucoproteins by the method of Uriel and Grabar [11]. Designation of peaks given in text.

somewhat faster than albumin, and fraction 2 with a mobility equal to that of albumin is very clear. The mobilities of fraction 4 and 5 with clear boundaries respectively equal the mobilities of α_1- and α_2-globulin. Fractions 6, 7 and 8 are in close contact with each other. They are found immediately around the start and they make up about 1/3 of all the protein extract (in mobility they are close to β_1-globulin). In almost all cases fraction 8 agrees with the β_2-globulin fraction of serum and is composed of two difficultly separable fractions, 8a and 8b. Fraction 9 in electrophoretic mobility occupies a middle place between β_2- and γ-globulin of serum. Fractions 10, 11 and 12, which in some cases seem the same, have the electrophoretic mobility of serum γ-globulin. In quantitative respects, fraction 10 predominates among them.

The relative content of proteins of each fraction is given in the table. Satistical treatment makes clear that difference between the protein fractions of the starved and fed cats does not occur. We may suggest that the coefficient of variation for fractions 2-5 (with mobility of serum albumin and α-globulin) is higher than in the other fractions.

Staining for glucoproteins shows the presence of polysaccharide components in the protein fractions 2-4, 6-8 and 10 (figure; curve III).

DISCUSSION OF RESULTS

The electrophoretograms of the extracted proteins from gastric mucosa of cats differ qualitatively and quantitively from the electrophoretograms of Demling and co-workers [4]. We find twice as many protein fractions in them; prealbumin and proteins with the mobility of serum γ-globulin are relatively less in amount, and proteins with the mobility of serum albumin are relatively more. Probably these differences are chiefly to be explained by the difference in the methods of obtaining the extracts and their separation, and also by differences in the species studied. Experiments with the gastric mucosa confirm the relatively higher separating power of electrophoresis on agar as compared to paper electrophoresis, which was shown earlier for liver proteins. Also, our process of

extraction with the use of butanol according to the principle of Morton [12, 13] allows the extraction of a greater part of the protein bound in the lipoprotein complexes of the microsomes and mitochondria, while extraction with dilute salt solutions extracts only the soluble proteins of the cytoplasm.

In the resulting extracts of gastric mucosa there are less proteins with the mobility of serum albumin than in blood serum.

The comparatively high coefficient of variation for the different protein fractions includes experimental errors, but also indicates the relatively high lability in amounts of soluble proteins of the gastric mucosa. The presence of glucoproteins in the fractions 2-4, 6-8 and 10 shows that in these, perhaps, there are proteins related to stomach secretions. These fractions predominate in a quantitative respect. For localization of the secretory proteins we must analyze the enzyme activity of the different fractions.

SUMMARY

In extracts of the proteins of cat gastric mucosa obtained by the method of Kaplanskii and co-workers (n-butanol and 2% solution of NaCl) and submitted to electrophoresis on agar, we have shown 13 well formed protein fractions. We have described two fractions with a higher mobility than serum albumin. Protein with the mobility of blood serum β-globulin predominates, after which come proteins with the mobility of serum albumin, and β- and γ-globulins, and we have shown the presence of glucoproteins.

We have not shown a qualitative or even quantitative difference between the protein fractions in extracts of the gastric mucosa of starved and fed cats.

LITERATURE CITED

[1] D. Northrop, M. Kunitz and R. Herriott, Crystalline Enzymes (IL, Moscow, 1950) [Russian translation].

[2] M. Fossel, Z. Physiol. Chem. 282, 164 (1947).

[3] R. Willstatter, F. Haurowitz and F. Memmen, Z. Physiol. Chem. 140, 203 (1924).

[4] L. Demling, H. Kinzelmeir and N. Z. Henning, Ges. Exptl. Med. 122, 416 (1954).

[5] A. Cresseri, Boll. Soc. ital. biol. sperim. 30, 718 (1954).

[6] D. Nachkov and O. Nachkova, Bull. Soc. chim. biol. 41, 159 (1959).

[7] V. Neuhoff, Arzneimittel-Forsch. 9, 607 (1958).

[8] S. Kaplanskii and S. B. Kuzovleva, Biokhimiya 22, 162 (1957).*

[9] P. Grabar and C. A. Williams, Biochim. et biophys. acta 17, 67 (1955).

[10] K. V. Giri, Naturwissenschaften 43, 36 (1956).

[11] J. Uriel and P. Grabar, Ann. Inst. Pasteur 90, 427 (1956).

[12] R. K. Morton, Nature 166, 1092 (1950).

[13] R. K. Morton, Methods in Enzymology, vol. 1, Ed. Colowick S. P. and N. O. Kaplan, (N.Y., 1955).

[14] A. Ilkov and T. Nikolov, Vopr. Med. Khimii 5, 358 (1959).

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THE EFFECT OF ETHANOL AMINE AND SOME OF ITS DERIVATIVES ON PHOSPHATASE ACTIVITY

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As investigations in our laboratory have shown, ethanol amine and some of its derivatives take an active part in phosphorus metabolism [1-4]. We have established that under the action of ethanol amine, diethanol amine, triethanol amine, choline, and phosphoethanol amine in different organs there is a change in the content of total phosphorus and also of its separate fractions. As a result of the introduction of these amines into the animal organism we found an increase in content of such important substances as ATP, phosphocreatine, phospholipids, etc.[5, 6].

Alkaline and acid phosphatases play an important part in phosphorus metabolism; therefore we were interested in studying the activity of these enzymes under the influence of these amines. The literature contains a number of reports of change in activity of phosphatases under the influence of various factors [7-14].

Among the amines there has been a study of the effect on phsophatase activity of diethanol amine, and it has been shown that its injection into white mice causes increased activity of the alkaline phosphatase of the liver, but does not affect the acid phosphatases [14].

METHODS

In long and short term experiments we studied the action of ethanol amine, diethanol amine, triethanol amine, choline, and phosphoethanol amine on phosphatase activity in the blood and organs of animals. In the long term experiments we studied phosphatase activity in the blood plasma of dogs (four dogs). In the short term experiments we studied phosphatase activity in liver, kidney, and brain of white rats (30 rats, five for each substance). The amines were injected subcutaneously in neutral form, in the amount of 5 mg/kg. Phosphatase activity was determined by the hydrolysis of glycerophosphate [15] at pH 5.0 and 9.2. Homogenates of the organs were prepared in a Potter type glass homogenizer. Blood was taken from the dogs immediately before injection of the amines and 30 and 60 min after the injection. Rats were killed by decapitation 60 min after injection of the amines.

RESULTS

The data obtained from one of the animals (Table 1) shows that in control experiments the phosphatase activity of blood plasma is not changed, while under the influence of ethanol amine the activity of alkaline and acid phosphatases is markedly decreased. An analogous effect is shown by diethanol amine and phosphoethanol amine; the first acts somewhat more strongly on the alkaline phosphatases. Triethanol amine and choline have almost no effect on phosphatase activity. Analogous results were also obtained for the other dogs.

The results given in Table 2 show that under the influence of ethanol amine phosphatase activity in the liver is increased somewhat, and in the kidney and brain it is decreased. Phosphoethanol amine has an analogous action. Diethanol amine causes strong increase in activity of alkaline phosphatase in the liver while the activity of acid phosphatases is almost unchanged. The decreased phosphatase activity in kidney and brain under the influence of diethanol amine is much greater than under the influence of the other amines. Triethanol amine

TABLE 1

Effect of the Amines Studied on Phosphatase Activity in Dog Blood Plasma

Period of analysis, min	Control		Ethanol amine		Diethanol amine		Triethanol amine		Choline		Phospho-ethanol amine	
	I*	II*	I	II	I	II	I	II		II	I	II
0	2,3	0,6	2,2	0,7	2,1	0,6	2,2	0,6	2,0	0,6	2,0	0,7
30	2,3	0,6	1,6	0,5	1,1	0,3	2,1	0,5	1,9	0,6	1,7	0,6
60	2,3	0,6	1,1	0,2	0,8	0,3	2,2	0,5	1,9	0,5	1,0	0,3

*I) alkaline phosphatase; II) acid phosphatase.

TABLE 2

Phosphatase Activity in Rat Organs in Bodansky Units

Liver		Kidney		Brain	
I*	II*	I	II	I	II
		Control			
0,65	2,95	22,50	3,75	1,02	0,85
0,71	2,75	20,64	3,68	0,78	0,76
0,66	2,88	23,19	3,90	0,84	0,73
0,62	2,98	22,47	3,64	0,88	0,70
0,60	2,80	19,37	3,60	0,77	0,62
		Ethanol amine			
0,84	3,34	18,55	2,85	0,78	0,67
0,72	3,07	19,04	2,97	0,72	0,62
0,72	3,08	16,28	2,78	0,70	0,70
0,84	3,54	18,73	3,09	0,68	0,54
0,80	3,26	14,93	3,17	0,64	0,56
		Diethanol amine			
1,37	2,95	11,25	2,66	0,52	0,40
1,25	2,84	14,64	2,68	0,48	0,34
1,08	2,87	12,70	2,50	0,44	0,48
0,92	2,68	10,85	2,36	0,39	0,51
1,10	2,75	12,27	2,36	0,40	0,44
		Triethanol amine			
0,66	2,94	20,15	2,98	0,76	0,67
0,72	3,15	21,58	2,66	0,83	0,49
0,82	2,90	19,67	2,75	0,66	0,58
0,72	3,09	23,10	3,10	0,72	0,56
0,79	3,00	18,00	2,61	0,69	0,57
		Choline			
0,80	3,08	22,50	2,96	0,72	0,60
0,85	3,10	21,15	3,10	0,69	0,55
0,81	3,25	20,68	3,08	0,78	0,52
0,68	3,04	21,13	2,74	0,78	0,73
0,76	2,80	19,57	2,64	0,64	0,50
		Phosphoethanol amine			
0,74	3,30	17,60	3,00	0,58	0,63
0,65	3,28	19,57	2,92	0,68	0,55
0,71	3,14	18,80	2,86	0,86	0,72
0,80	2,96	18,56	2,85	0,74	0,63
0,69	3,08	18,86	3,18	0,81	0,74

*I) alkaline phosphatase; II) acid phosphatase.

and choline act like ethanol amine, but somewhat more weakly, especially with regard to the alkaline phosphatases; it is interesting that they have almost no action on the alkaline phosphatase of the kidney while the acid phosphatases of kidney and brain are affected somewhat more strongly by triethanol amine and choline than by ethanol amine.

DISCUSSION

Our previous investigations [1, 4] showed that under the influence of ethanol amine the reabsorption of phosphorus in the kidney canals is somewhat strengthened and 2-3 hours after its injection into dogs the excretion of phosphorus in the urine is decreased about 100 fold. Since the concentration of inorganic phosphorus in the blood then increases comparatively little, we suggested that part of the phosphorus was retained in various organs. Further studies [2] with radioactive phosphorus (P^{32}) confirmed this suggestion. It was found that in liver, along with accumulation of phosphorus there was increased synthesis of a number of phosphoorganic compounds, especially phospholipids [16, 18].

We showed [4, 6] that diethanol amine in greater degree than ethanol amine and other amines increases reabsorption of phosphorus in the kidney canals and increases the amount of phospholipids in the liver. We can believe that increased phosphatase activity under the influence of the amines, especially ethanol amine and diethanol amine, permits the synthesis of phospholipids by liberating phosphoric acid from its esters for the synthesis of the phospholipids.

It is known that phosphatase activity in the liver has definite value for the transformation of glycogen into glucose by splitting phosphoric acid from glucose-1-phosphate. This can explain the increase of glucose in the blood under the influence of ethanol amine [19]. Decreased phosphatase activity in the kidney under the influence of ethanol amine and diethanol amine can permit retention of phosphate esters which are found in the organism with these amines. Evidently in the presence of these amines phosphorus undergoes reverse absorption from the kidney canals in the form of the corresponding esters; hence, retention of these esters strengthens reabsorption of phosphorus.

It is known that in rickets the activity of alkaline phosphatase in blood plasma is greatly increased. The introduction of vitamin D causes decreased activity of this enzyme [20]. Decreased activity of alkaline phosphatase in blood plasma under the action of ethanol amine on the one hand, and on the other the fact of increased reabsorption of phorphorus in the kidney canals and its accumulation in various organs, including the bones, [2], permits the suggestion that ethanol amine has antirachitic properties. Diethanol amine and phosphoethanol amine in all probability have the same properties.

Our results agree with the literature data that the main source of the phosphatase of blood plasma is the kidney [21]. In our investigation we have observed a parallel between phosphatase activity of the kidney and the blood plasma. Thus, ethanol amine and phosphoethanol amine decrease the phosphatase activity in the kidney and blood plasma; diethanol amine, which decreases the phosphatase activity of the kidney more than these amines, acts in the same way on blood plasma phosphatase; finally, triethanol amine and choline are almost inactive for the alkaline phosphatase of kidney and blood plasma.

SUMMARY

Ethanol amine, diethanol amine, and phosphoethanol amine decrease the activity of alkaline and acid phosphatases in blood plasma; triethanol amine and choline have almost no action on these enzymes. Almost all of the amines which we studied increase phosphatase activity in the liver equally and lower it in the kidney and brain; diethanol amine does not affect the activity of acid phosphatase of liver, and triethanol amine and choline do not affect activity of kidney alkaline phosphatase. Diethanol amine acts on phosphatase activity more strongly than do the other amines.

LITERATURE CITED

[1] G. V. Kamalyan and G. V. Barsegyan, Trudy Erevanskogo Zoovet. In-ta 20, 9 (1956).

[2] G. V. Kamalyan and G. V. Barsegyan, Biokhimiya 22, 971 (1957).

[3] G. V. Kamalyan, Trans. All-Union Conference on Noninfectious Diseases of Agricultural Animals, p. 5 (1957).

[4] G. V. Bersegyan, Trudy Erevansk. Zoovet In-ta. 21, 25 (1957).

[5] G. V. Kamalyan, M. G. Gasparyan and L. V. Davtyan, Doklady Akad. Nauk Arm. SSR 27, 87 (1958).

[6] G. V. Kamalyan, M. G. Gasparyan and G. V. Barsegyan, Doklady Akad. Nauk Arm. SSR 27, 295 (1958).

[7] A. Aschkenasy, V. Abitbol and M. Piette, Compt. Rend. Soc. Biol. 150, 1168 (1956).

[8] S. Oeriu, L. V. Balanescu, L. Buruina, E. Hadarag and P. Niculescu, Studii si cercetari indocrinol. Acad. R.P.R. 6, 447 (1955).

[9] J. B. Marsh and D. L. Drabkin, Biol. Chem. 168, 61 (1947).

[10] T. P. Bharadwaj, J. Andrews and D. S. Nadkaini, Ind. J. Med. Sci. 10, 646 (1956).

[11] E. N. Slesareva, Izv. Timiryazevsk. S-kh. Akad. 1, 145 (1957).

[12] M. Dumont, Presse Med. 65, 535 (1957).

[13] M. B. Bel'chikova, Zdravookhr. Sov. Latvii 2, 209 (1954).

[14] E. Annau and A. Manginelli, Nature 166, 816 (1950).

[15] A. Bodansky, J. Biol. Chem. 101, 93 (1933).

[16] A. P. Platt and R. R. Porter, Nature 160 (4078), 905 (1947).

[17] C. Artom and W. E. Cornatzer, J. Biol. Chem. 176, 949 (1948).

[18] G. V. Kamalyan and G. V. Barsegyan, Trudy Krevansk. Zoovet. In-ta. 22, 31 (1957).

[19] G. V. Kamalyan, Trudy Erevansk. Zoovet. In-ta. 14, 65 (1952).

[20] H. F. De Luca and H. Steenbock, J. Biol. Chem. 222, 937 (1956).

[21] D. L. Drabkin and J. B. Marsh, J. Biol. Chem. 171, 455 (1947).

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THE ACTION OF A SURFACE ACTIVE SUBSTANCE ON THE ENZYME SYSTEM WHICH OXIDIZES MALIC ACID IN CYTOPLASMIC MEMBRANES OF MICROCOCCUS LYSODEIKTICUS

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It was showed earlier that the oxidation of malic acid with absorption of oxygen is found in only one fraction of the lyzate of the bacteria Micrococcus lysodeikticus, namely, in the cytoplasmic membranes, the "ghosts" [1, 2]. According to the data of Bruemmer et al. [3] this process in Azobacter vinelandii involves pyridine nucleotide dehydrogenase, flavoprotein enzymes, and cytochromes. However, there is some doubt as to the role of the pyridine nucleotide dehydrogenase since Dohn [4] has found in Micrococcus lysodeikticus along with malic dehydrogenase a particular enzyme which causes dehydrogenation of malic acid without reduction of DPN. Although the composition of the enzyme which produces oxidation of malic acid in bacterial cells has still not been studied enough, the data on the localization of this process in the cytoplasmic membranes suggests the presence of a definite special organization of this enzyme. Destruction of the membrane structure should cause a corresponding destruction in the coordination system of the enzyme. According to the data of a number of authors the membranes are complexes of proteins, lipids, and carbohydrates in which a number of enzymes are fixed [5]. Therefore treatment of the membranes with surface active substances which destroy the complexes and change the membrane structure is a possible path for studying the organization of enzymatic processes in it.

In the present work we have studied the effect of an anionic surface active substance, Neopol T, on the oxidation of malic acid by fractions of the lyzate of the bacteria Micrococcus lysodeikticus. Preparation of the lyzate was carried out in the presence of magnesium ions in order to obtain a preparation with the most active system for oxidizing malic acid.

METHODS

In the experiments we used a culture of the bacteria M. lysodeikticus, Fleming strain. The growth of the bacteria and collection of the bacterial mass were carried out by the method previously described [6]. Lysis was carried out for 30 min at 37° in 0.001 M phosphate buffer, pH 6.8, with content of 2.5 mg $MgSO_4$, 50 µg of lysozyme, and 40 mg of bacterial mass per ml (the dry weight was 25-30% of the bacterial mass). Determination of oxygen absorption, and reduction of DPN and 2,6-dichlorophenol indophenol (DPI) were run as described earlier [1, 2], but the amount of lyzate in the samples with DPI was 0.1 ml. Nitrogen was determined by micro-Kjehldahl, total carbohydrates by the anthrone reaction, nucleic acids according to Spirin [7]. Mannose was used as the standard in the anthrone reaction [8]. Lipids were determined in the lyophilized material after treatment with boiling absolute ethyl alcohol, distillation off of the solvent, and then extraction of the mixture with ligroin (to 60°) and absolute ethyl alcohol 5:1. The total amount of lipids was found by difference in weight of the material before and after treatment. Absorption spectra were studied in an SF-4 spectrophotometer. The color produced in the anthrone reaction was measured in an FÉK-M photoelectric colorimeter with a red light filter. The anionic surface active substance Neopol T was used in aqueous solution at a concentration of 10 mg/ml. We added 0.1 ml of Neopol T solution to 1 ml of lyzate and kept it with slight agitation at 30° for 30 min.

TABLE 1

Action of Neopol T on Oxygen Absorption and Reduction of DPI in Lyzate Fractions

Fraction	Microliters O_2/20 min per 4 mg of starting bacteria		ΔE after 1 min		ΔE after min/mg nitrogen	
	without Neopol T	with Neopol T	without Neopol T	with Neopol T	without Neopol T	with Neopol T
Lyzate	25	2,5	0,330	0,042	3,00	0,38
Membranes	28	—	0,300	0,005	15,00	0,50
Supernatant liquid	0,45	—	0,025	0,020	—	—

TABLE 2

Action of Neopol T on Malic Dehydrogenase Activity Measured by DPI Reduction in Lyzate Fractions

Fraction	ΔE after min/40 mg of starting bacteria		ΔE after min/mg nitrogen	
	without Neopol T	with Neopol T	without Neopol T	with Neopol T
Lyzate	0,053	0,050	0,048	0,045
Membranes	0,011	0,004	0,052	0,060
Supernatant liquid	0,047	0,046	—	—

Fractionation of the lyzate was carried out by centrifuging at 22000 g. The membrane precipitate was washed twice with 0.001 M phosphate with a content of 2.5 mg $MgSO_4$ per ml. For determination of activity the material was added to a volume of starting lyzate.

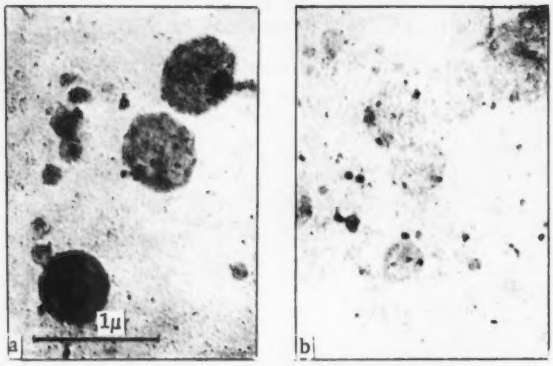
Preparation and examination of the material was carried out by the methods described previously [9] on an UEM-100 electron microscope in the Laboratory of Electron Microscopy of the Academy of Sciences, USSR, Moscow.

EXPERIMENTAL RESULTS

Fractionation of the lyzate showed that all the system for oxidation of malic acid with absorption of oxygen was localized in the cytoplasmic membranes (Table 1). In the supernatant liquid obtained after separation of the membranes, oxygen absorption due to malic acid oxidation was not found at all. When the lyzate which contained the membranes was treated with Neopol T, oxygen absorption was 85-90% suppressed, which indicates a break in the enzyme chain at one or more of its links. In order to determine how the treatment with the surface active substance was reflected on the enzymes which transfer hydrogen, we measured the reduction of the DPI and DPN in the lyzate treated with Neopol T and in control lyzates, and also in fractions obtained after centrifuging the lyzate.

The enzyme system which reduces DPI is thus chiefly bound in the fraction of the cytoplasmic membranes (Table 1).

In the supernatant liquid after removal of the membranes we observed slight reduction of DPI as compared to the full lyzate. The specific activity of the enzyme system which reduces DPI in the membranes is quite marked, which shows the localization of the system in the membranes. Treatment of the lyzate with Neopol T suppresses the reduction of DPI in the same degree to which it suppresses the absorption of oxygen. As a result of the action of the surface active substance there is destroyed in the membrane fraction the coordinated action of the enzymes which reduce DPI by oxidation of malic acid. The rate of reduction of the acceptor of the supernatant liquid after treatment of the lyzate with Neopol T is not changed. Hence, removal of the membrane from the lyzate or its treatment with an anionic surface active substance results in the same degree of suppression of reduction of DPI and absorption of oxygen.



Effect of treating the lyzate with Neopol T on the structure of the cytoplasmic membrane: a) membrane from lyzate without Neopol T; b) membrane from lyzate with Neopol T.

TABLE 3

Effect of Neopol T on Absorption by the Supernatant Liquid at 450, 280 and 260 mμ after Separation of the Cytoplasmic Membrane. Supernatant liquid diluted 1:50, except E_{450}.

Variant	E_{450}	E_{280}	E_{260}
Without Neopol T	0,097	0,181	0,317
With Neopol T	0,345	0,232	0,410

TABLE 4

Effect of Treatment of Lyzate with Neopol T on Composition of Cytoplasmic Membrane. In percent of dry weight of starting bacteria

Substance determined	Without Neopol T	With Neopol T
Protein	53,75	17,32
Nucleic acid	12,2	0,68
Carbohydrate	9,8	4,88
Lipid	20	16

Differing from the enzyme systems which reduce DPI, malic dehydrogenase, whose action is measured by reduction of DPN, is chiefly in the supernatant liquid which results after separation of the membrane fraction. The membrane fraction itself has slight activity (Table 2).

The specific activity of malic dehydrogenase in the membrane and in the full lyzate before fractionation is the same, which shows the equality of distribution of the enzyme in protoplasm. Since malic dehydrogenase is an individual enzyme and its chief mass is not bound with the large structural elements of the cell, Neopol T does not show any influence on its activity. After the experiments to show the action of Neopol T on the enzyme systems which reduce DPI, we ran analyses of the membrane fraction. The yield of membrane was 20% of the weight of the starting bacteria from the lyzate not treated with Neopol T, and about 8% from the lyzate which had been acted upon by the detergent. Corresponding to the decreased yield of membrane fraction there was a change in absorption of the supernatant liquid at 450, 280 and 260 mμ (Table 3), which showed a transformation in solution of proteins, pigments, and nucleic compounds. Direct analysis of the membrane fraction (Table 4) showed that in it, along with the proteins, lipids, and carbohydrates there was some quantity of nucleic acids. As a result of treatment with Neopol T the composition of the membrane fraction was changed. A considerable amount of proteins and nucleic acids went over to the soluble state. The amount of lipids which dissolved was about 20% of the original content of them in the membrane. Photographs under the electron microscope (figure) indicate the change in morphology of the membranes – they lose their definite contours and become transparent.

DISCUSSION OF RESULTS

The process of oxygen absorption due to oxidation of malic acid in the lyzate from M. lysodeikticus is stopped after treatment with a surface active substance, Neopol T. The reason for this inhibition in the first place is that there is a sharp slowing of the process of hydrogen transfer as measured by the reduction of 2,6-dichlorophenol indophenol. The sensitivity of the process of transfer of hydrogen to destruction of the structure depends on the fact that the enzymes which carry on this process are localized in the cytoplasmic membranes. Destruction of the structure and change in the composition of the membranes under the influence of Neopol T causes a break in the enzyme chain which transfers hydrogen, and destroys the coordination between the individual enzymes. The surface active substance does not act on the activity of the specific enzyme malic dehydrogenase.

An analogous effect of inhibition of oxygen absorption with simultaneous disruption of the hydrogen transport

chain was found by us in experiments in which lyzates were treated with DNA-ase [9]. It is evident that DNA has distinct value for maintaining the special organization of the enzymes which are localized in the cytoplasmic membrane. In this connection, it is a fact that the membrane preparations contain about 12% nucleic acid. According to the data of other authors [8, 10] the content of nucleic acids in the membranes is very low, and only Vennes [11] considers that preparations of the membrane of B. megaterium contain about 11% nucleic acids.

Comparison of the specific activity of the systems which oxidize malic acid, as measured by oxygen absorption or by reduction of DPI with the specific activity of malic dehydrogenase which uses DPN as a coenzyme shows a "contradictory" distribution of these enzymes in the cells of the bacteria studied. This finding suggests that malic dehydrogenase either is completely absent from the enzyme systems localized in the membranes, or occurs in these structures in such a state that exogenous DPN is not reduced. According to the data of Cohn [4] in M. lysodeikticus along with malic dehydrogase which requires DPN, there is an enzyme which dehydrogenates malic acid without the participation of the coenzyme. This enzyme is localized in the cytoplasmic membranes. Thus, evidently, a chain of enzymes which produce dehydrogenation of malic acid and further transfer of hydrogen toward oxygen is localized in the cytoplasmic membrane and its action is destroyed when the membrane structure is destroyed.

SUMMARY

The cytoplasmic membrane is obtained by lysis of the bacteria M. lysodeikticus. The membrane fraction contains about 20% of the weight of the starting bacteria. We find in the membrane composition 53.75% protein, 20% lipids, 12.2% nucleic acids, and 9.8% carbohydrates. All the system of enzymes which oxidize malic acid with absorption of oxygen are localized in the cytoplasmic membrane. The absorption of oxygen stops as a result of treatment by an anionic surface active agent, Neopol T. This inhibition of malic acid oxidation is related to the rupture of the chain of enzymes which transfer hydrogen as is shown by the change in rate of reduction of 2,6-dichlorophenol indophenol by the fraction of cytoplasmic membrane after treatment with Neopol T. The problem of the nature of dehydrogenating enzyme systems requires further study, since the activity of the malic dehydrogenase which uses DPN is slight in the membrane.

LITERATURE CITED

[1] A. I. Oparin, N. S. Gel'man, L. G. Zhukova and M. A. Lukoyanova, Biokhimiya 23, 909 (1958).*

[2] N. S. Gel'man, L. G. Zhukova, M. A. Lukoyanova and A. I. Oparin, Biokhimiya 24, 481 (1959).*

[3] J. Bruemmer, P. Wilson, J. Glenn and F. Crane, J. Bacteriol. 73, 113 (1957).

[4] D. Cohn, J. Biol. Chem. 233, 299 (1958).

[5] N. S. Gel'man, Uspekhi Sovrem. Biol. 47, 152 (1959).

[6] A. I. Oparin, N. S. Gel'man and L. G. Zhukova, Doklady Akad. Nauk SSSR 105, 1036 (1955).*

[7] A. S. Spirin, Biokhimiya 23, 656 (1958).*

[8] A. Gilby, A. Few and K. McQuillen, Biochim. et biophys. acta 29, 21 (1958).

[9] N. S. Gel'man, L. G. Zhukova and A. I. Oparin, Doklady Akad. Nauk SSSR 126, 198 (1959).*

[10] C. Weibull and L. Bergström, Biochim. et biophys. acta 30, 340 (1958).

[11] J. Vennes, cited by [10].

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A COMPARATIVE STUDY OF THE TRANSAMINATION REACTIONS OF DICARBOXYLIC AMINO ACIDS AND THEIR AMIDES IN CANCER OF THE HUMAN STOMACH

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Since the characteristic peculiarity of tumors, mostly malignant, is their intensive growth, which is closely related to new protein formation, the problem of biosynthesis of proteins and the study of the characteristics of nitrogen metabolism in tumors is very real in modern biochemistry and oncology.

In a previous communication [1] we considered the theoretical grounds for a study of the transamination reaction in tumors as an important reaction of amino acid metabolism, and gave the results of experiments on transamination of dicarboxylic amino acids and their amides in the tissues of a number of transplanted tumors and malignant tumors of the human stomach. However, in view of the small number of experiments with the human stomach cancer tissues (only 2 experiments) and the absence of histological characteristics of these tumors, it was impossible to draw any conclusions as to the specific characteristics of the transamination reaction in the various histological sections of the stomach cancers.

The problem of the present study is to explain to what extent the transamination enzyme systems are destroyed in histological sections of various human stomach cancers; whether to accept the position of Cohen [2] as to the existence of an inverse relation between transaminase activity in tumors and the rate of protein synthesis in stomach cancers; and, finally, what is the relative rate of transamination of dicarboxylic amino acids and their amides in tumor tissue.

EXPERIMENTAL

Plan of Experiments and Method of Investigation

In this work we have used human stomach cancers (adenocarcinoma and scirrhus) and gastric mucosa not attacked by cancer. As controls we also used gastric mucosa removed because of pyloric stenosis or gastric or duodenal ulcers.*

The experiments were run with fresh tumor sections and gastric mucosa under aerobic conditions. We added 13.3 μmoles of ketoacid and 6.6 μmoles of amino group donor or NH_4Cl to 50 mg of tumor sections. The control samples contained only the ketoacid or only the amino acid (volume of sample, 1 ml). The samples were incubated in a phosphate buffer, 1/15 molar, for 2 hours at 37°. The protein was precipitated by boiling the sample, cooling, and then adding TCA (final concentration 4.5%) and separating by centrifuging. The supernatant liquid was used for chromatographic analysis. The dicarboxylic acids formed in transamination were determined by the method of Paskhina [3].

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TABLE 1

Formation of Aminodicarboxylic Acids in Sections of Gastric Mucosa and Tumor Tissue from Ammonia and α-Ketoglutaric and Oxaloacetic Acids by Reductive Amination

Increase of aspartic acid		Increase of glutamic acid		Increase of aspartic acid		Increase of glutamic acid		Increase of aspartic acid		Increase of glutamic acid		Increase of aspartic acid		Increase of glutamic acid	
mg	% amination	mg	% amination	mg	% amination	mg	% amination	mg	% amination	mg	% amination	mg	% amination	mg	% amination
normal				in scirrhus				in adenocarcinoma				scirrhus		adenocarcinoma	
0,21	1,1	0,17	0,9	0,17	0,9	0,21	1,2	0,19	1,0	0,20	1,1	0,05	0,3	0,19	1,1	0,10	0,5	0,12	0,7
0	0	0	0	0	0	1,40	8,0	trace	0	0,74	4,3	trace	0	2,19	12,3	trace	0	0,49	2,8
trace	0	1,33	7,6	0	0	0,49	2,8	trace	0	1,19	6,8	0	0	0,94	5,3	0	0	1,66	9,5
0	0	trace	0	0	0	0,16	0,9	trace	0	2,13	12,1	0	0	0,08	0,4	trace	0	1,44	8,2
trace	0	trace	0	0	0	0,37	1,9	0	0	0,76	4,3	0	0	0,79	4,0	0	0	0,53	3,0
0	0	0,87	4,9	0	0	0,75	3,1	trace	0	1,24	7,1	0	0	0,89	4,7	0	0	1,68	9,5

A sample of 30 microliters of protein-free filtrate was deposited on a strip of filter paper (Whatman No. 1) and also a sodium bicarbonate-neutralized (to bromothymol blue) solution of glutamic or aspartic acid. As the solvent we used phenol saturated with water in an atmosphere of ammonia. The chromatogram was removed after 18-20 hours, dried in air to full removal of phenol, and developed with a 0.5% solution of ninhydrin in acetone. After a day's development the separate spots of the amino acids appeared. The spots were cut from the paper and the color was eluted with 3 ml of 50% ethyl alcohol for 20 min; then studied with a photometer against a blank sample (eluate from a part of the paper through which solvent had passed) using an FÉK-M photocolorimeter. Calculation of the amount of amino acids formed by transamination was carried out on a graph calibrated for pure amino acids.

The activity of the transamination reaction was expressed by the increased formation of amino acids after subtraction of the control values and was calculated per g of starting tissue. We used α-ketoglutaric acid and oxaloacetic acid as acceptors of the NH_2 group; for NH_2 group donors we used the L-isomers of glutamic and aspartic acids, glutamine, and asparagine.

RESULTS

Altogether we ran 32 experiments. The results (Table 1) show that in normal mucosa sections and also in sections of mucosa not affected by the tumor in adenocarcinoma and scirrhus, reductive amination of oxaloacetic acid does not occur. On the other hand, in all mucosa studied, especially in adenocarcinoma, amination of α-ketoglutaric acid with formation of glutamic acid occurs, although the percent amination is small (from 3.1 to 7.6). The data on reductive amination of ketoacids in preparations of tumor tissue show that ammonia in the presence of oxaloacetic acid does not increase the amount of tissue aspartic acid; at the same time, in the presence of α-ketoglutaric acid, the increase in glutamic acid under the influence of ammonia is 4.6 and 5.6% of the total volume of amination in the tissues of scirrhus and adenocarcinoma, respectively.

In Table 2 we give data on the synthesis of aminodicarboxylic acids by transamination in sections of mucosa under different conditions, and also in sections of tumor tissue. These results indicate that, in normal mucosa, glutamine and asparagine in only a few cases increase the growth of dicarboxylic amino acids, while glutamic and aspartic acids are transaminated intensively with the corresponding ketocarboxylic acids (28.4 and 20.4% transamination). In distinction to this, when we study the transamination activity of intact portions of gastric mucosa in the tumorous process (scirrhus and adenocarcinoma)

TABLE 2

Synthesis of Aminodicarboxylic Acids by Transamination in Sections of Gastric Mucosa and Tumor Tissues

Increase in glutamic acid from α-ketoglutaric acid and				Increase in aspartic acid from oxaloacetic acid and			
asparagine		aspartic acid		glutamine		glutamic acid	
mg	transamination, %	mg	transamination, %	mg	transamination, %	mg	transamination, %
Normal mucosa							
0,13	0,7	2,51	14,3	1,31	6,8	2,07	10,7
0	0	4,76	27,1	0	0	6,13	31,8
2,49	14,2	7,32	41,7	1,66	8,6	7,19	37,4
trace	0	3,66	20,8	trace	0	1,66	8,6
trace	0	5,33	30,4	0	0	2,66	13,8
trace	0	6,30	45,9	trace	0	3,90	20,3
Mucosa in scirrhus							
0,11	0,6	2,36	13,4	1,37	7,1	1,1	5,7
0,34	1,7	8,65	49,3	1,37	7,1	8,32	43,2
0,21	1,2	6,47	36,8	0,24	1,2	4,52	23,5
0,14	0,8	1,90	11,3	0,75	4,3	0,92	5,2
0,17	0,9	4,50	23,4	1,13	6,4	3,72	19,3
0,26	1,3	3,96	20,6	0,97	5,5	4,15	23,6
Mucosa in adenocarcinoma							
0,15	0,8	2,70	14,6	1,0	5,3	1,80	8,4
0,49	2,8	5,40	30,9	2,0	10,4	4,40	22,8
2,13	12,1	7,90	45,5	2,60	13,8	8,30	43,2
2,56	14,6	8,0	45,9	1,50	7,6	4,41	22,8
0,32	1,8	4,10	23,5	0,40	2,1	0,80	4,5
2,25	12,8	5,30	30,3	2,10	11,1	3,74	19,2
Scirrhus							
0,14	0,8	2,0	11,6	1,07	5,6	1,20	6,2
0,22	1,1	7,30	41,7	2,10	10,0	10,60	55,3
trace	0	5,70	29,6	0,84	4,4	6,20	32,4
0	0	1,60	9,4	1,35	7,7	7,80	44,4
0,12	0,52	4,21	21,8	1,64	9,3	5,37	30,6
0	0	6,20	32,2	1,42	8,1	7,20	41,1
Adenocarcinoma							
0,19	1,1	2,60	15,0	1,30	6,7	1,50	8,1
0,38	2,2	4,11	23,5	2,0	10,4	4,40	22,8
3,20	18,4	6,52	36,8	0,44	2,3	7,90	41,3
3,20	18,4	7,60	44,6	1,66	8,6	5,90	31,2
0,78	4,4	4,63	26,4	0,79	4,1	0,94	4,9
1,33	6,6	4,64	26,6	3,90	20,7	4,42	22,8

we find that glutamine and asparagine increase the yield of aminodicarboxylic acids by transamination in all experiments. However, in this series of experiments, the aminodicarboxylic acids are many times more active (from 2-20 fold) than the corresponding amides in transamination with ketoacids.

The study of formation of aminodicarboxylic acids by transamination in the tumor tissues themselves showed that this process occurs both in scirrhus and adenocarcinoma, and glutamine and asparagine are transaminated with the corresponding ketodicarboxylic acids at about the same intensity only in sections of adenocarcinoma, while in scirrhus tissue asparagine scarcely increases the amount of tissue amino acids, and glutamine increases the aspartic acid in this tumor. Glutamic and aspartic acid transaminate several times more intensively (2-5 fold) in both tissues than do the corresponding amides. Also, glutamine reacts by transamination with oxaloacetic acid in all the tissues studied (Table 2), although the relative percentage of transamination is small (5.5-8.8%). Asparagine under these conditions transaminates with α-ketoglutaric acid only in sections of adenocarcinoma and gastric mucosa in adenocarcinoma (7.5-8.6%). Glutamic and aspartic acids transaminate

intensively in stomach tissues, and in scirrhus the transamination reaction shifts to the right, while in adenocarcinoma and in tissues of the gastric mucosa the reaction shifts to the left according to the equation:

$$\text{glutamic acid} + \text{oxaloacetic acid} \rightleftharpoons \frac{1}{2}\ \text{aspartic acid} + \alpha\text{-ketoglutaric acid}$$

Addition of ammonium salts increases the yield of glutamic acid in all tissues when it is incubated with α-ketoglutaric acid, while its incubation with oxaloacetic acid scarcely increases the amount of tissue aspartic acid.

DISCUSSION OF RESULTS

A number of authors [4-8] have observed weak transamination activity or the absence of this activity in the tissues of some experimental tumors, which does not agree with the idea of the dominating role of the transamination reaction in nitrogen metabolism, and hence in the synthesis and breakdown of proteins in the tissues. As is known, the distinctive peculiarity of tumors, especially malignant ones, is their uncontrollably rapid growth, that is, in tumors, anabolic reactions proceed at a high rate with new formation of proteins. Considering the very wide distribution of transaminases in biological materials, the study of the transamination reaction in tumor tissues seems to us to be fully justified. The fact of the observation of an active glutamic-aspartic transaminase in our experiments with human stomach tumors agrees with the high protein synthesizing function of malignant tumor cells.

However, this fact contradicts the position of Cohen [2] on the existence of an inverse relation between the rate of synthesis of proteins and the activity of the glutamic-aspartic transaminase in rapidly growing tissues, including tumors. The results of Kitu and Awapara [9] directly confirm our results. These authors have observed highly active glutamic-aspartic and glutamic-alanine transaminases in tissues of rat and mouse lymphosarcoma. It is also very valuable in this work to compare the transaminase ability of normal lymphatic tissue with that of the cancerous tissue, since often conclusions of the absence or weak activity of transaminases in tumors are drawn on the basis of comparisons of their activity with the activity of transaminases in the liver or pectoral muscle of the pigeon.

It is difficult to explain in our experiments the very weak process of transamination of glutamine and asparagine in the tumor tissues and in the "normal" gastric mucosa (amides 2-16 times less active than the dicarboxylic amino acids). In all probability, under the conditions of the investigation, the amino groups of the amides do not transfer to α-ketoacids, for if they did, the amides would transaminate with the same speed or intensity as the aminodicarboxylic acids. Perhaps (though we have not performed balance experiments which would permit us to settle this question) under the experimental conditions, the amide group of glutamine and asparagine is taken up in the protein of the tissue without preliminary hydrolysis by a transamidation reaction. However, such a reaction should occur more slowly than a transamination reaction, since the remaining dicarboxylic acid could be intensively transaminated with additional α-ketoacid.

Such a possibility has recently been demonstrated by Levintow [10] using labeled N^{15}-glutamine in experiments with cells of He La tumors. He showed that the amide nitrogen of $N^{15}H_2$-glutamine is taken up in the amide nitrogen of the protein of HeLa cells without preliminary hydrolysis to free ammonia, and the labeling appears neither in the α-aminoacids of the protein nor as ammonium chloride. This suggested to the author that ammonia nitrogen like the amide nitrogen of glutamine is not a precursor of α-amino nitrogen of proteins. These data are an indirect confirmation of our experiments in which addition of ammonium salts resulted in only their slight use for the synthesis of amino acids from α-ketoacids. All these investigations are included in the general ideas developed by Braunshtein [11] and Euler [12] on the predominating role of the transamination process in the synthesis of amino acids in the living organism.

Thus, our data permit us to conclude that aminodicarboxylic acids are transaminated in the tissues studied with greater speed (2-16 times more intensively) than are their amides. The ideas of Cohen on the inverse relation between rate of synthesis of proteins in rapidly growing tissues (including tumors) and the activity of the glutamic-aspartic transaminase cannot be applied to stomach cancers.

SUMMARY

Aminodicarboxylic acids are transaminated in sections of "normal" mucosa and in tumors of the stomach 2-16 times as intensively as the amides.

We have confirmed the previous study on the presence of active glutaminase I in human gastric cancer; we have observed weak asparaginase activity in sections of adenocarcinoma and mucosa not attacked by cancer (in adenocarcinoma); in sections of scirrhus and "normal" mucosa under these experimental conditions asparaginase has not been observed.

Glutamine gives a transamination reaction with oxaloacetic acid in all the tissues studied, although the relative percentage of transamination is small; asparagine under these conditions transaminates with α-ketoglutaric acid only in tissues of adenocarcinoma and of mucosa not attacked by cancer in adenocarcinoma.

The reductive amination of oxaloacetic acid with added ammonia is not found under these experimental conditions; α-ketoglutaric acid undergoes direct reductive amination, but the yield of glutamic acid by this path is 5-6 times less than by transamination between aspartic acid and ketoglutarate. In gastric mucosa not attacked by cancer in scirrhus and adenocarcinoma there is more intense transamination reaction of dicarboxylic amino acids and their amides than in "normal" mucosa.

LITERATURE CITED

[1] T. T. Berezov, Vopr. Med. Khimii 4, 39 (1959).

[2] P. P. Cohen, G. L. Hekhuis and E. Sober, Advances Cancer Res. 2, 405 (1942).

[3] T. S. Paskhina, Biokhimiya 19, 702 (1954).

[4] H. Euler, H. Hellström, G. Gunther, L. Elliot and S. Elliot, Z. Physiol. Chem. 259, 201 (1939).

[5] A. E. Braunshtein and R. M. Azarkh, Biokhimiya 5, 1 (1940).

[6] F. Cedrangolo and G. Carandante, Arch. Sci. Biol. 27, 6 (1941).

[7] P. P. Cohen and G. L. Hekhuis, J. Biol. Chem. 140, 711 (1941).

[8] P. P. Cohen and G. L. Hekhuis, Advances Cancer Res. 1, 620 (1941).

[9] S. Kitu and J. Awapara, Advances Cancer Res.13, 694 (1953).

[10] L. Levintow, Science 123, 611 (1957).

[11] A. E. Braunshtein, Biochemistry of Amino Acid Metabolism,(Izd. AMN SSSR, Moscow, 1949).

[12] H. Euler, H. Hellström et al., Z. Physiol. Chem. 259, 201 (1939).

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CHLOROPHYLL SENSITIZED OXIDATION-REDUCTION REACTIONS OF PYRIDINE NUCLEOTIDES IN CHLOROPHYLL SOLUTIONS AND HOMOGENATES OF PLANT LEAVES

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At the present time, ideas of the photochemical reduction of pyridine nucleotides (PN) as a step in the photosynthetic process have spread widely.

In 1949 we showed experimentally the possibility of photosensitized reduction of diphosphopyridine nucleotide (DPN) in solution from the energy of light absorbed by chlorophyll [1]. In 1951 appeared the work of Vishniac and Ochoa [2, 3], Tolmach [4], and Arnon [5] which showed the possibility of reduction of PN in chloroplast homogenates. In 1956, San Pietro [6, 7] demonstrated the reduction of PN in homogenates by a spectrophotometric method and found a protein factor which catalyzed the light reaction. Arnon and co-workers [8] came to the same conclusions. Vernon obtained similar results for homogenates of photosynthesizing bacteria [9]. Frenkel observed reduction of PN by the use of reduced flavin mononucleotide in chromatograms of photosynthesizing bacteria [10].

In a number of studies [6-8] the reduction of PN was measured by increase in the absorption coefficient at 340 mμ after illumination and the fall in this value after introduction of alcohol-dehydrogenase and acetaldehyde as a substrate for oxidation. Measurement of the absorption spectrum of the reduced form of PN in these studies was not successful, apparently because of strong light absorption by the homogenates in this portion of the spectrum. Duysens and Amesz [11] found reduction of PN directly in the living plant leaf, using the method of differential spectroscopy and measuring the fluorescence. In the work of one of us with Litvin [12], measurement of the fluorescence spectrum of plant leaves at low temperature permitted observation of maxima which corresponded to bound and free forms of reduced PN.

In the present work we have posed the following problems:

1. To explain the relation of the sensitized photoreduction of DPN in solution (model system) with the reduction of this compound in plant leaf homogenates during progressive destruction of the natural condition of the pigments under the action of pyridine and heat.

In the model system with increasing amounts of water the chlorophyll goes from true to colloidal solution, while the addition of pyridine to green leaf homogenate leads to gradual destruction of the natural state of the granules — destruction of the coordination of the photochemical and enzymatic reactions [13] with final solution of the chlorophyll.

2. To establish the possibility of sensitization by chlorophyll of the photooxidation of reduced PN in solution and in leaf homogenates and to study this process which is probably connected with the use in the organism of the reduced form of PN.

Chlorophyll Sensitized Reduction of PN

In our first study [1] in order to establish the reduction of DPN we measured the absorption spectra of

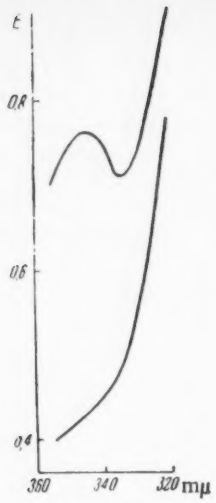

Fig. 1. Chlorophyll sensitized reduction of TPN. Absorption spectrum: 1) before illumination; 2) after illumination.

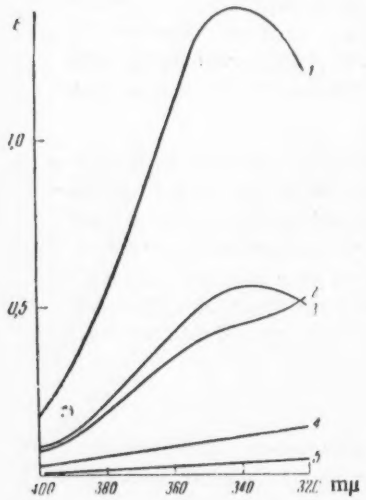

Fig. 2. Chlorophyll sensitized reduction of DPN. Experiments carried out in a vacuum: 1) in illumination; 2) in the dark; 3) in air with illumination; 4) illumination without ascorbic acid; 5) illumination without DPN.

pyridine solutions which contained chlorophyll, ascorbic acid, and DPN (at 320 and 400 mμ) before and after illumination. The measurements were carried out directly in a Thunberg vacuum tube; they were made with considerable difficulty since there was great absorption of light by the pyridine and the reaction components in this region of the spectrum.

In the present work we have measured the absorption spectra of the reacting systems in lined cuvettes (length of optical path d = 0.15 cm), pouring into them the contents of the vacuum tubes before and after illumination. The experimental procedure was the following: we placed in a vacuum tube 3 ml of a solution of chlorophyll a or a + b in pyridine (absorption coefficient E at 670 mμ and d = 1.0 equal to 3-5), 4 mg of DPN* or 3 mg of triphosphopyridine nucleotide (TPN) dissolved in 0.6 ml of a 4% aqueous solution of ammonia, and 4 mg of ascorbic acid. A part of the solution was put into the cuvette, the absorption spectrum was measured from 320 to 400 mμ and it was put back into the tube which was evacuated and illuminated for 5 min at 15° with red light (light filter KS-13) of 10^4 erg/cm^2/sec. After illumination, the solution from the tube was again measured in the cuvette. Figure 1 shows that illumination in red light of the absorption maximum of chlorophyll produces a maximum absorption which corresponds to the reduced form of PN. Control experiments (experiments without illumination or experiments with a reaction mixture of the composition chlorophyll-DPN or TPN and chlorophyll-ascorbic acid) did not show maximum absorption in this part of the spectrum.

However, along with the appearance of an absorption maximum there was a general rise in the spectrum curve, evidently due to absorption in this region of the spectrum by oxidation products of ascorbic acid and of reduction of chlorophyll.

In order to find only the reduction of PN we also used another method based on the removal of DPN after reaction. In this case the experimental procedure was as follows: we placed in a Thunberg vacuum tube 4 ml of chlorophyll a or a + b in pyridine (E at 670 mμ about 2), 10 mg of ascorbic acid, and 10 mg of DPN in 1 ml of 4% aqueous ammonia solution. The air was removed from the tube with a vacuum pump with boiling of the solvent for 3 min, after which the tube was illuminated at 15° by a cinema lamp of 300 watts through a condensor and light filter KS-13 for 20 min. After the reaction mixture had been illuminated, it was poured into five times the volume of acetone cooled by dry ice to − 10°. The PN then precipitated and was filtered on a glass filter. The precipitate was carefully washed free of pyridine by cold acetone and dissolved in 5 ml of water. The absorption spectrum of the resulting solution was measured in an SF-4 spectrophotometer.

Figure 2 shows the results of the experiments carried out under different conditions. The chlorophyll sensitized reduction of DPN occurred only in the presence of ascorbic acid on illumination and was more evident in a vacuum than in the presence of air. We should note that the reaction also goes in the presence of oxygen of the air. Probably oxygen is quickly reduced with photosensitization and thus is removed from the reaction medium, after which photoreduction of PN occurs.

*In this work we used preparations which contained 38-44% DPN obtained by us from yeast by the usual method. Such a preparation contained chiefly the oxidized form of DPN. The preparation of TPN contained about 60% of pure compound.

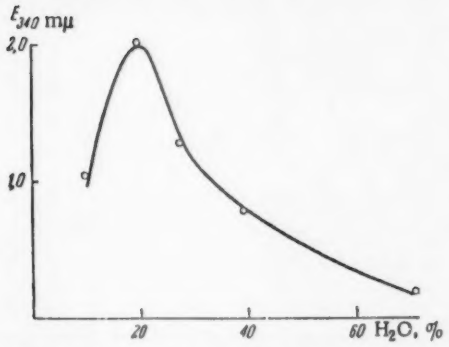

Fig. 3. Dependence of photoreduction of DPN on amount of water in the reaction mixture.

The same effect also occurs in photoreduction of chlorophyll by ascorbic acid in the presence of air with weak illumination; there is an induction period which corresponds to the removal of oxygen from the reaction medium by photosensitized oxidation which uses up part of the ascorbic acid in the system. After this there is photoreduction of the chlorophyll. We studied the dependence of photoreduction of DPN on amount of water in the pyridine (from 10 to 70% water content). The chlorophyll concentration was constant ($2 \cdot 10^{-5}$ M) for the whole series of experiments. The amounts of DPN and ascorbic acid were 5 mg per 1.5 ml of chlorophyll solution (in mixtures of pyridine and water). With increase in the amount of water in the pyridine above 50% there was a gradual decrease in fluorescence and aggregation occurred, formation of colloidal particles of chlorophyll, which reached a maximum at the minimum amount of pyridine. The dependence of amount of DPN reduction on amount of water in the reaction mixture (under the same experimental conditions) is shown in Fig. 3, which indicates that the greatest amount of reduction of DPN occurs at 20% water. With 10% water the effect is somewhat lower, which may be connected with the smaller solubility of DPN. Actually, under these conditions there is formation of the red photoreduced form of chlorophyll while at 20% water, because of the rapid occurrence of the reverse reaction, the photoreduced form of chlorophyll does not appear; the green color of the solution does not change.

With further increase in amount of water (70%) there is decreased ability for reduction which is evidently connected with the gradual aggregation of the chlorophyll. We should remark that the obvious absorption maximum of reduced DPN is obtained at 10-30% water in the reaction mixture. At 50% water this maximum is less clear. At 70% water the absorption spectrum differs little from the control experiments in the dark.

Chlorophyll Sensitized Reduction of DPN in Green Leaf Homogenates

The experiments were carried out with leaf homogenates of beans, beets, spinach, and wheat seedlings. All the plants were grown in a greenhouse. Ten g of leaves were ground in the cold with 20 ml of phosphate buffer, pH 7.0. The suspension was pressed through a cloth and centrifuged for 5 min at 1000 rpm. We used the homogenate which contained fragments of chloroplasts and granules.

In these experiments it was not expedient to use the method of removal of DPN since in the homogenates, as distinguished from the pyridine solutions, there was rapid enzymatic oxidation of the reduced DPN, and so the measurements had to be made in a vacuum directly after illumination. For running these experiments we used a special Thunberg tube with narrow plane parallel walls (d = 0.15 cm and working volume about 1 ml) which permitted following the reaction by spectrophotometric measurements.

The experimental procedure was as follows: 4 mg DPN was dissolved in 0.1 ml of 4% ammonia solution and treated with 0.9 ml of leaf homogenate or its mixture with pyridine. We kept the same chlorophyll concentration in the experiments (E at 670 mμ 0.6-0.8 at d = 0.15 cm).

After evacuation of the air we measured the absorption spectrum in the range from 320 to 360 mμ through a KS-13 light filter for 20 min and measured the absorption spectrum after which we passed air and again measured the absorption spectrum.

In the leaf homogenates, in agreement with the experiments of San Pietro, we found the absorption maximum of the reduced form of PN. The addition of pyridine inhibited this reaction. With 10% pyridine we could still find some reduction of DPN, while 70% pyridine dissolved the chlorophyll and fully destroyed its ability for this reaction. In the case of addition of ascorbic acid to the homogenate (5 mg in 1.5 ml), reduction of DPN was inhibited. Perhaps this effect is connected with some increased acidity of the medium. In order to test this, we ran experiments in which we used sodium ascorbate instead of ascorbic acid. Photoreduction of DPN in homogenates which did and did not contain ascorbate was similar.

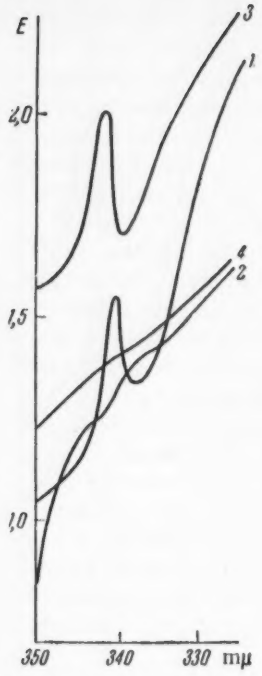

Fig. 4. Oxidation of reduced DPN photosensitized by chlorophyll in solution and in green leaf homogenates. Absorption spectra: 1) homogenate before illumination; 2) after illumination; 3) chlorophyll solution before illumination; 4) after illumination.

Determination of reduction of DPN (by change in optical density at 340 mμ) under conditions of illumination and depending on the amount of pyridine added (%) gave the following results:

Added	0%	5%	10%	70%
DPN + ascorbic acid	0	0	0,230	0,960
DPN	0,160	0,080	0,060	0
DPN + sodium ascorbate	0,130	—	—	—

Thus, addition of pyridine in experiments with ascorbic acid increases the reduction of DPN occurring in the light, reaching a maximum when chlorophyll dissolves in pyridine.

We should remark on the following fact. In our experiments in the presence of 5% pyridine the ability of the homogenate to reduce DPN is retained, while the ability to give the Hill reaction is completely lost with this amount of pyridine [13]. Evidently, the photoreduction of DPN and its control by enzyme systems is more stable to the action of pyridine than is the complex of enzymes which take part in the Hill reaction.

The absorption maximum of chlorophyll is almost unchanged in the experiments with homogenates. The distinct absorption maximum of reduced DPN was observed in experiments with ascorbic acid in the range from 10 to 70% pyridine. In experiments without ascorbic acid a distinct absorption maximum was found only if the reaction mixture contained not over 5% pyridine. In this case, reduction of DPN occurs because of hydrogen donors which are different from ascorbic acid.

Thus, destruction of the natural state of binding permits the occurrence of the simpler reaction, photoreduction of DPN due to ascorbic acid, while without the addition of outside hydrogen donors the ability to reduce DPN is lost when the natural state of the granules is destroyed.

Chlorophyll Photosensitized Oxidation of Reduced Pyridine Nucleotides

As is known, chlorophyll can sensitize the oxidation of a number of compounds. However, until now, as far as we know, there has been no study of the ability of PN to take part in this reaction.

In our experiments a solution of reduced DPN was prepared as follows: 10 mg of 44% DPN was dissolved in 0.5 ml of water. To the solution was added 0.5 ml of 1% $NaHCO_3$ and 0.5 ml of 5% sodium hydrosulfite in 1% $NaHCO_3$ solution. After standing for 20 min at room temperature, the solution was treated with 2.5 ml of a mixture of equal volumes of 1% solutions of $NaHCO_3$ and Na_2CO_3 [14]. The unreacted hydrosulfite was removed by passing air through the solution, and complete destruction of the hydrosulfite was controlled by the spectrophotometric method. The amount of reduced DPN was determined by the value of the absorption coefficient at 340 mμ.

Oxidation of the reduced DPN was studied in solutions of chlorophyll and in homogenates of green leaves, determined by the change in the optical density at 340 mμ and by measurement of the absorption of oxygen in the oxidation of DPN.

Photooxidation of DPN-H in Chlorophyll Solutions. A water solution which contained 1 mg of reduced DPN in 1 ml of liquid was mixed with a solution of chlorophyll a + b in alcohol or pyridine in the ratio 1:5. The absorption coefficient E at 665 or 670 mμ was equal to 0.6-0.7.

The solution was placed in a Thunberg tube and illuminated in the presence of oxygen of the air for 20 min through a red KS-13 filter. Figure 4 shows the results of spectrophotometric measurement (in the range 320-350 mμ) of solutions of chlorophyll in pyridine in the presence of reduced DPN before and after illumination.

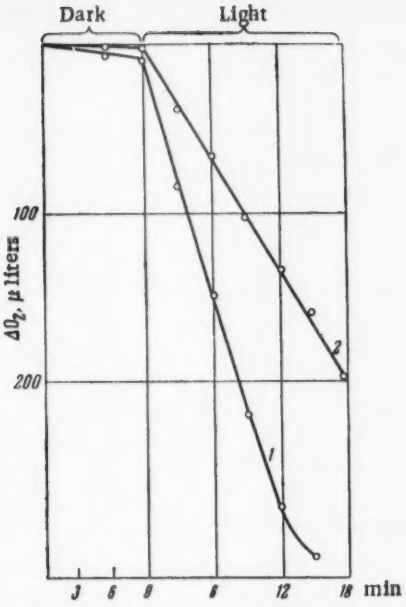

Fig. 5. Chlorophyll sensitized photo-oxidation of DPNH and ascorbic acid in alcohol solution: 1) DPNH; 2) ascorbic acid.

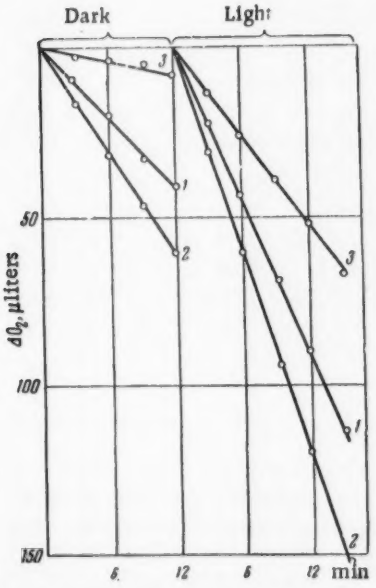

Fig. 6. Photosensitized oxidation of reducted DPN with different homogenate fractions: 1) homogenate; 2) supernatant liquid; 3) chloroplasts.

The results show that with illumination the maximum of reduced DPN disappears because of oxidation. Use of the Warburg apparatus shows that under the conditions of illumination of solutions of chlorophyll in alcohol with red light in the presence of reduced DPN there is rapid absorption of oxygen. The reaction does not occur in the dark. The chlorophyll concentration in these experiments was 10^{-5} M, the concentration of reduced DPN $1.5 \cdot 10^{-4}$ M; the experimental results are given in Fig. 5. For comparison we show the oxidation of ascorbic acid ($2 \cdot 10^{-3}$ M) sensitized by the same chlorophyll solution.

Photo-oxidation of reduced DPN by lead homogenates. The experiments were carried out by spectrophotometric and manometric methods. We studied the activity of unseparated homogenates of the leaves of various plants, and fractions: chloroplasts and supernatant liquid. The leaves were ground at 0° with 1/15 M phosphate buffer, pH 7.0, containing 10% sucrose, pressed through cloth, centrifuged at 1000 rpm, then at 5000 rpm, and washed several times with the same buffer. For the experiments we took homogenate after the first centrifuging, the supernatant liquid after the second centrifuging, and the chloroplasts after washing with phosphate buffer.

Spectrophotometric measurements. In these experiments we used leaf homogenate. To 0.9 ml of homogenate in phosphate buffer, pH 8.0, we added 0.1 ml of the reduced DPN solution. The absorption spectra of the reaction mixtures were photographed in the range 320-360 mμ and they were illuminated with red light of 104 erg/cm^2/sec for 20 min. At the end of the illumination the maximum of reduced DPN had fully disappeared (Fig. 4).

Study of the activity of fractions by the manometric method. The experiments were carried out in the Warburg apparatus. In the experiment we took 9 ml of homogenate or fraction, which contained 10^{-5} M chlorophyll, and 1 ml of reduced DPN solution $1.5 \cdot 10^{-4}$ M. First (12 min) we measured the rate of the dark, enzymatic oxidation of reduced DPN, and then illuminated (18 min) with red light through a KS-13 filter and measured the rate of sensitized oxidation.

A comparative study of the fractions showed that the rate of oxidation with illumination in unseparated homogenate and supernatant liquid was much higher than in the chloroplasts. However, the relative rate of photochemical oxidation (total rate of reaction with illumination after subtracting rate of enzymatic oxidation) was higher in the chloroplast fraction (Table 1). Heating to 60° completely inhibited the enzymatic oxidation and lowered the rate of photochemical oxidation considerably (Table 2).

When the fractions stood for a long time in the dark and cold (1°), from 1 to 7 days, the rate of enzymatic oxidation increased, approaching the rate of the process in light, which scarcely changed.

The addition of 5% pyridine greatly decreased the rate of enzymatic and photochemical oxidations. However, if more pyridine was added, dissolving the chlorophyll, the rate of photosensitized oxidation increased to the level which we found in the true chlorophyll solutions (Table 3).

TABLE 1

Photooxidation of DPN-H by Fractions of Leaf Homogenates, Rate of Reaction Expressed in Microliters of Oxygen Absorbed per min. L) experiment in light; D) experiment in dark; $\Delta = L - T$

Leaves of plant	Homogenate			Supernatant liquid			Chloroplasts		
	L	D	Δ	L	D	Δ	L	D	Δ
Beet	10,0	3,6	6,4	10,7	4,8	5,9	9,6	0,7	8,9
Bean	6,7	5,1	1,6	5,2	4,1	1,1	4,8	1,1	3,7
Corn	9,5	5,6	3,9	—	—	—	7,9	1,9	6,9

TABLE 2

Effect of Heating (to 60°) on Rate of Oxidation of DPN-H by Homogenate Fractions. Designations as in Table 1

Leaf of plant	Homogenate			Supernatant liquid			Chloroplasts		
	L	D	Δ	L	D	Δ	L	D	Δ
Beet	2,9	0,7	2,2	3,0	0,5	2,5	2,0	1,0	1,0
Bean	4,7	1,1	3,6	5,3	1,3	4,0	2,2	0,7	1,5
Corn	1,8	0,5	1,3	—	—	—	3,7	1,3	2,4

TABLE 3

Effect of 5% Pyridine on Rate of Oxidation of DPN-H by Homogenate Fractions. Designations as in Table 1

Leaf of plant	Homogenate			Supernatant liquid			Chloroplasts		
	L	D	Δ	L	D	Δ	L	D	Δ
Beet	4,0	1,7	2,3	6,2	1,5	4,7	2,3	0,7	1,6
Bean	4,2	3,5	0,7	4,4	3,9	0,5	1,7	0,7	1,0
Corn	2,0	0,5	1,5	—	—	—	2,3	0	2,3

When a 0.02 M solution of potassium cyanide was added, the light and dark reactions were fully inhibited.

We see from these experiments that the property of photosensitizing the oxidation of reduced DPN lies to a considerable degree in the chloroplasts, more than in the other fractions. When pyridine is added, the ability for the light reaction is retained while the enzymatic oxidation is fully inhibited. This indicates that the light reaction is related to a more stable enzyme system.

DISCUSSION

These experiments show that chlorophyll sensitized reduction of PN by ascorbic acid with storage of light energy (E_0 of ascorbic acid 0.05 v; E_0 of PN 0.32 v) can occur in solutions of chlorophyll and in biologically disorganized systems — suspensions of chloroplasts and granules in the presence of pyridine. In intact homogenates, hydrogen donors other than ascorbic acid are used for reduction of PN; in these processes specific proteins take part [6-8]. It is likely that elementary photoreduction of PN which occurs with participation of chlorophyll is

accompanied by later enzymatic reactions. We should mention in this connection that there is an observed correlation between activity of pyridine nucleotide dehydrogenase and the photochemical activity of the homogenate [15].

However, it is difficult to judge accurately as to the evolution of the oxygen of water in these reactions, since the newly reduced forms of PN are very energetically oxidized by enzymatic and photochemical processes. In this connection we must explain the significance of the photosensitized oxidation of PN when there is photophosphorylation in the chloroplasts as observed by Arnon [8].

In our previous work we repeatedly argued that photosensitized reactions of this type were the basis for the reversible photochemical reduction of chlorophyll: here the hydrogen donor molecule which gives up an electron (hydrogen) to the excited chlorophyll molecule in these experiments is ascorbic acid or reduced DPN, and the electron (hydrogen) acceptor, which reacts in the dark (with photosensitized chlorophyll) is PN or oxygen [16].

SUMMARY

In this work we have studied the photoreduction of DPN and TPN and the photooxidation of reduced DPN in solutions of chlorophyll and in homogenates of the green leaves of plants.

In chlorophyll solutions DPN is reduced only in the presence of ascorbic acid, while in homogenates of plants reduction occurs in the absence of this compound. With destruction of the natural state of the granules by pyridine, the ability to reduce DPN is lost, but there is retention of the ability to carry out a simpler photochemical process, the transfer of hydrogen from ascorbic acid to DPN, which reaches a maximum with the greatest amount of pyridine. Thus, in homogenates this reduction is connected with enzymatic steps which use hydrogen donors other than ascorbic acid.

We have observed that chlorophyll in solution and in homogenates is capable of energetic photosensitized oxidation of reduced pyridine nucleotides. This "pyridine nucleotide photooxidase" activity of homogenates is suppressed by heating and by the action of pyridine.

Student of the M. V. Lomonosov Moscow State University T. N. Krasil'nokova participated in the experimental work.

LITERATURE CITED

[1] A. A. Krasnovskii and G. P. Brin, Doklady Akad. Nauk SSSR 67, 325 (1949).

[2] W. Vishniac and S. Ochoa, Nature 167, 768 (1951).

[3] W. Vishniac and S. Ochoa, J. Biol. Chem. 195, 75 (1952); Symp. Phosphorus Metabolism 2, 467 (1952).

[4] L. Tolmach, Arch. Biochem. 33, 120 (1951); Nature 167, 946 (1951).

[5] D. L. Arnon, Nature 167, 1008 (1951).

[6] A. San Pietro, Science 124, 118 (1956).

[7] A. San Pietro and N. M. Lang, J. Biol. Chem. 231, 211 (1958).

[8] D. L. Arnon, F. Watley and M. Allen, Nature 180, 182 (1957); Science 177, 1026 (1958).

[9] L. P. Vernon and E. D. Ihnen, Biochim. et biophys. acta 24, 115 (1957).

[10] A. M. Frenkel, J. Amer. Chem. Soc. 80, 3479 (1958).

[11] L. M. N. Duysens and J. Amesz, Biochim. et biophys. acta 24, 19 (1957).

[12] F. F. Litvin and A. A. Krasnovskii, Doklady Akad. Nauk SSSR 120, 764 (1958).*

[13] L. M. Kosobutskaya and A. A. Krasnovskii, Biokhimiya 18, 340 (1953).

[14] S. Gutcho and E. Stewart, Analyt. Chem. 20, 1185 (1958).

*Original Russian pagination. See C.B. Translation.

[15] N. M. Sisakyan, A. A. Krasnovskii, E. S. Mikhailov and G. N. Brin, Biokhimiya 18, 626 (1952).

[16] A. A. Krasnovskii, Zh. Fiz. Khimii 30, 968 (1956); J. chim. phys. et phys.-chim. biol. p. 968 (1958).

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THE AMINO ACID COMPOSITION OF WHEAT GRAIN PROTEINS

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At the present time a large amount of work has accumulated which is devoted to the study of the qualitative and quantitative amino acid composition of the total protein of wheat grains and of the separate protein fractions [1].

However, the data which are given for the content of the same amino acid in wheat grain proteins are often very different.

We have not been able to locate data on the presence in wheat grain globulins and albumins of isoleucine, serine, threonine, cystine, and methionine. The most complete information on amino acid composition is for gliadin. Exact data on the amino acid composition of wheat proteins will have great value for many problems of chemistry and biochemistry of proteins, and also for nutrition.

There is also very great interest in the changes in amino acid composition in wheat proteins depending on conditions of growth and biological characteristics of the varieties. Changes in the content of some amino acids in the proteins of seeds and other organs of plants depending on internal and external factors have been studied by a number of authors [2-7]. As to data on changes in amino acid content in separate fractions of wheat grain proteins under the influence of growth conditions and the relation of characteristics of varieties, we have been unable to find these in the literature.

Thus, it follows that in spite of the long period of investigation of many questions of the chemistry and biochemistry of wheat proteins from both the practical and theoretical viewpoints, the studies are still not sufficient.

In the present work we give data on the composition and amounts of the separate amino acids in gliadin, albumin, and globulin of different sorts of wheat depending on conditions of growth and biological characteristics of the varieties.

MATERIALS AND METHODS

We studied the varieties of wheat grown in different regions of the Armenian SSR, "Érinatseum" (a summer soft variety) from Leninakan and from Basargechar and "Galgalos" (a summer soft variety from Leninakan and from Sukhoi-Fontan).

The soil and climatic conditions for the growth of these varieties of wheat are given in Table 1, based on data from the Armenian Water Service and from Agadzhanyan et al. [8]. The regions in which the chosen varieties of wheat were grown differed from each other both in type of soil and in climatic conditions (Table 1). It is important to note that the climatic conditions in growing the materials for investigation were typical for these regions. For more complete characteristics of the conditions of growing the varieties which we studied, we give data on the agricultural conditions under which they were raised.

"Érinatseum" variety, summer soft. Leninakan Selection Station (Shirak agricultural zone); height above

TABLE 1

Soil and Climatic Conditions of Some Regions of Armenia

Region	Type of soil	Mean annual temperature of air, °C		Amount of precipitation, mm		Average temperature of air (°C) and amount of precipitation (mm) in the growing period of wheat by months*								
						1953		1954						
		1953	1954	1953	1954	XI	XII	I	II	III	IV	V	VI	VII	VIII
Leninakan	Weakly carbonated black earth	5,3	6,0	414	487	−2,0 33,3	−13,2 16,2	−12,0 39,1	− 9,8 32,2	3,7 37,7	+5,0 42,9	+11,9 122,1	+15,8 35,3	+20.4 30,8	+14,8 15,0
Basargechar	Mountain chesnut	4,2	4,5	661	324	−2,5 57,3	−11,8 15,8	− 8,6 26,7	10,8 96	−5,0 16,3	+3,2 19,5	+9,3 37,7	+14,0 21,9	+16,6 43,1	+17,2 8,5
Sukhoi-Fontan	Black earth	4,8	4,0	744	709	−2,2 98,2	− 9,6 17,6	− 7,3 65,5	− 6,5 56,4	−1,3 93,9	+4,0 108,0	+11.1 119,9	+14,8 91,5	+18,0 40,7	+19,5 25,4

*In the numerator is given the temperature (°C); in the denominator, the precipitation (mm).

sea level 1600 m; climate, mountainous cold. Sowed on April 12, 1954 in a field of sainfoin, harvested in autumn. Normal sowing 170 kg/hectare. In the autumn with basic plowing the following fertilizers were added: superphosphate 3 centner/hectare; ammonium sulfate 1.5 centner/hectare; potassium chloride 1 centner/hectare. Watered 45 days after sowing. Cultivated in the spring with harrowing. Harvested August 20, 1954 at the stage of full ripeness. Yield 22 centner/hectare.

Basargechar Agricultural Station (agricultural zone of the Sevansk basin); height above sea level 2000 m, dry continental climate. Sowed April 27, 1954 in a field of winter wheat, plowed in the autumn. Soil fertilized in the autumn with superphosphate (60 kg/hectare) and in the spring before sowing with ammonium sulfate (2.3 centner/hectare); norm for sowing, 170 kg/hectare. Harvested August 25, 1954 at the stage of full ripeness. Yield 15 centner/hectare.

"Galgalos" ("Delphi") variety, summer soft. Sukhoi-Fontan Seed Cultivation Collective (Aparano-Akhtinsk agricultural zone); height above sea level 1550 m; climate moderately warm. Sowed May 20, 1954 in a field of winter wheat. Sowing norm, 200 kg/hectare. Soil before sowing fertilized with superphosphate (2 centner/hectare). A month after sowing superphosphate (1 centner/hectare) added. Harvested August 10, 1954 at the stage of full ripeness. Yield 12 centner/hectare.

Leninakan Agricultural Station (Shirak agricultural zone). Conditions of raising the "Galgalos" variety in this region were the same as for the "Erinatseum" variety.

Thus, our choice of types of wheat permits comparison in the study of their biochemical properties of the results for the same type of wheat grown under different conditions and for different kinds of wheat grown under the same conditions.

The preparation of the proteins from the whole ground grains of the wheat varieties was as follows: albumin and globulin by the method of Pence and Elder [9] with some modifications; gliadin by the method of Kretovich [10]. Determination of the composition and content of separate amino acids from the protein preparations was carried out by the method of paper chromatography [11]. Separation of the amino acids was carried out on "Leningrad slow" paper in three solvent systems: n-butanol-acetic acid-water (4:1:1), phenol buffered with 0.068 M phosphate buffer, pH 12 (1:1), and o-cresol- buffered with phosphate buffer, pH 6.2 (1:1). In these solvent systems we obtained clear separation of the individual amino

TABLE 2

Amino Acid Content in Gliadin Depending on Conditions of Growing the Wheat. In % dry weight of protein

Amino acids	Type and region of growth					
	"Galgalos"			"Erinatseum"		
	Leninakan	Sukhoi-Fontan	Difference, %*	Leninakan	Besargechar	Difference, %*
Arginine	3,25	3,79	14,20	4,55	3,72	22,3
Histidine	3,08	2,72	13,2	3,54	2,50	40,0
Lysine	1,20	0,80	50,0	0,80	0,70	14,2
Tyrosine	1,35	1,32	2,2	1,39	1,58	13,6
Tryptophan	0,70	0,80	14,2	0,67	0,77	14,9
Phenylalanine	2,27	3,32	37,4	3,74	2,66	40,4
Proline	12,30	9,00	36,6	13,00	12,00	8,3
Cystine + cysteine	1,90	2,51	32,1	2,90	2,05	41,4
Methionine	0,30	0,25	20,0	0,37	0,31	19,3
Serine	7,34	6,61	11,0	7,61	7,47	2,0
Threonine	1,22	1,02	19,6	0,94	0,83	13,2
Leucine + isoleucine	11,50	10,00	15,0	12,78	11,82	8,1
Valine	2,16	2,80	29,6	2,65	2,22	19,3
Glutamic acid	31,18	34,40	10,0	28,90	34,67	19,9
Aspartic acid	1,98	1,45	36,5	1,68	1,85	17,0
Glycine	5,77	5,39	7,0	9,19	7,08	30,0
Alanine	2,61	1,81	44,2	3,69	2,51	47,0
Amide nitrogen	4,20	4,38	4,2	4,69	3,96	18,4

*In calculating the difference in content of the individual amino acids, their minimum amount was taken as 100%.

acids [11]. The chromatograms were developed with 1% acetone solution of ninhydrin; the spots of the amino acids were eluted with 4 ml of 0.5% $CdCl_2$ solution in 40% ethanol. The intensity of color of the eluate was determined in a spectrophotometer (SF-4) at 510 mμ.

Tryptophan was determined in separate samples after hydrolysis with 14% $Ba(OH)_2$ by the method of Horn and Jones [12]. Proline was determined by the method of Archer et al. [13]. We should say that in the determination of cystine + cysteine it is necessary to use an aqueous or buffer (veronal-HCl, pH 7) solution of ninhydrin. For the determination of cystine, its spot, whose location had been determined previously on a control chromatogram strip, was cut out with scissors and soaked in 0.5 ml of a 1% aqueous solution of ninhydrin. The reaction was carried out at 105° for 10 min. The color was eluted with 4 ml of 40% ethanol at room temperature for 2 hours. The intensity of the color which developed was determined in a spectrophotometer (SF-4) at 550 mμ.

For determination of the amount of each individual amino acid we first constructed a standard curve. Then when we had measured orienting concentrations of amino acids in the material studied, using the standard curves we again carried out a chromatographic study of the samples, with simultaneous deposit on the same sheet of paper of three standard mixtures of amino acids. One of these mixtures contained amino acids which corresponded to the concentration previously found in the orienting study, and the concentration in the second mixture was 5% above and in the third mixture 5% below this value. The content of the amino acids in the material studied was determined by comparing the intensity of color of the eluate from the amino acid spots in the three mixtures with the intensity of color of the eluate from the spots of the corresponding amino acids in the material studied.

The difference between the amino acid concentration in the studied material and in the mixture of the pure preparations in the above three standard amino acid mixtures did not exceed ± 3%. Therefore the amount of amino acid in the mixture which most closely resembled the studied material was taken as its concentration in the latter.

Amide nitrogen was determined by the method described by Belozerskii and Proskuryakov [14].

TABLE 3

Amino Acid Content of Albumin in the Grain, Depending on Conditions of Growing the Wheat. In % dry weight of protein

Amino acids	Type and region of growth					
	"Galgalos"			"Erinatseum"		
	Leninakan	Sukhoi-Fontan	Difference, %*	Leninakan	Basargechar	Difference, %*
Arginine	7,52	6,30	19,3	8,18	7,61	7,4
Histidine	6,75	6,10	10,6	5,36	5,77	7,6
Lysine	2,76	2,77	0,3	3,37	3,21	4,8
Tyrosine	3,00	3,77	25,6	4,85	3,34	54,4
Tryptophan	2,55	2,00	27,5	2,10	2,45	16,6
Phenylalanine	2,76	2,91	5,4	2,50	3,16	24,0
Proline	1,64	2,20	23,1	2,00	2,40	20,0
Cystine + cysteine	2,40	3,10	25,0	2,10	2,80	33,3
Methionine	0,68	0,73	7,3	0,66	0,80	21,2
Serine	6,77	8,73	28,8	7,73	8,20	6,0
Threonine	2,10	2,97	40,4	2,61	2,00	30,5
Leucine + isoleucine	13,20	11,90	11,0	14,35	13,72	12,8
Valine	4,83	3,32	15,4	5,21	4,13	26,1
Glutamic acid	13,50	12,16	11,1	15,24	14,43	55,0
Aspartic acid	6,46	7,80	20,7	9,98	7,61	31,1
Glycine	13,46	11,89	13,2	9,71	12,28	26,5
Alanine	2,48	3,78	52,4	3,96	2,74	44,4
Amide N	1,33	1,20	10,8	1,25	1,15	8,6

*In calculating the difference in content of individual amino acids their minimum concentration was taken as 100%

TABLE 4

Amino Acid Content in Globulin of the Grain, Depending on Conditions of Growing the Wheat. In % of dry weight of protein

Amino acids	Type and region of growth					
	"Galgalos"			"Erinatseum"		
	Leninakan	Sukhoi-Fontan	Difference, %*	Leninakan	Basargechar	Difference, %*
Arginine	8,57	10,37	21,0	9,58	8,34	14,8
Histidine	4,81	6,67	38,6	6,28	5,90	64,4
Lysine	3,21	3,37	4,9	3,04	3,98	30,9
Tyrosine	3,23	3,14	2,8	4,82	3,42	40,9
Tryptophan	1,00	1,23	23,0	1,53	1,54	0,6
Phenylalanine	4,24	3,70	19,4	3,47	4,12	18,5
Proline	2,00	2,80	40,0	2,50	3,00	20,0
Cystine + cysteine	2,30	1,90	21,0	2,70	2,00	35,0
Methionine	1,23	1,03	19,4	1,73	1,38	25,3
Serine	7,14	8,60	20,4	9,35	7,38	26,7
Threonine	2,00	2,55	27,5	2,80	1,96	42,8
Leucine + isoleucine	13,71	13,42	2,1	13,08	12,80	26,8
Valine	6,00	6,16	16,2	4,26	5,32	24,6
Glutamic acid	14,00	13,15	6,4	13,11	12,53	4,5
Aspartic acid	7,81	8,25	5,6	9,57	7,45	28,4
Glycine	13,10	11,21	16,8	12,21	10,63	14,8
Alanine	4,00	3,84	4,2	4,67	4,16	12,2
Amide N	1,04	1,07	2,8	0,85	0,71	19,7

*In calculating the difference of content of individual amino acids their minimum concentration was taken as 100%.

RESULTS

In Tables 2, 3 and 4 we give data on the amino acid composition of the protein fractions from wheat grains and the amount of individual amino acids contained in them. All the studied preparations of gliadin, albumin, and globulin contained the same nineteen amino acids. Hence, these wheat proteins did not differ from each other in qualitative composition.

However, in gliadin, the glutamic acid content (28.90-34.67%) was much higher than in the other fractions of wheat protein. Proline was also present in greater amount in this protein (9-12%). These results agree fully with the results of other authors [15, 16]. On the other hand, gliadin preparations contain very small amounts of lysine, tryptophan, and threonine. The lysine content in our samples of gliadin from the various forms of wheat varied from 0.70 to 1.20%, of tryptophan, from 0.67 to 0.80%, and of threonine from 0.83 to 1.22%. In a small amount in gliadin preparations we also found aspartic acid (1.45-1.98%) and methionine (0.25-0.37%). This fact is perhaps because methionine is strongly decomposed in acid hydrolysis.

Table 3 shows that in albumin preparations the content of glycine, glutamic acid and the sum of leucine and isoleucine is relatively higher than the content of the other amino acids. In albumin preparations the glycine content varies from 9.71 to 13.46%, the sum of leucine and isoleucine, from 11.90 to 14.35%, and the glutamic acid from 12.16 to 15.24%. In albumin preparations the tryptophan content (2.00-2.55%) several times surpasses the content of this amino acid in gliadin (0.67-0.80%). This characteristic, along with some others, serves as a criterion for determining the purity of the isolated protein fractions [9]. The content of aspartic acid also is considerably higher in the albumin preparations than in the gliadin. Table 3 also shows the content of serine, methionine, threonine, isoleucine, and cysteine, the presence of which has not been described in the literature for the albumin of wheat grains.

It is evident from these results that, in the preparations studied, the albumin content of serine varies from 6.77 to 8.73%, of threonine from 2.00 to 2.97%, of methionine from 0.66 to 0.80%, of the sum of leucine and isoleucine from 11.90 to 14.35%, and of the sum of cystine and cysteine from 2.10 to 3.10. It should be noted that the data on the content of methionine in albumin and in gliadin may be somewhat low.

The data on the amino acid content in the globulin preparations from wheat grain are given in Table 4. Most of all, globulin contains glycine, glutamic acid, and the mixture of leucine and isoleucine. The glycine content in these preparations varies from 10.63 to 13.10%, glutamic acid from 12.53 to 14.00%, and the sum of leucine and isoleucine from 12.80 to 13.71%. We also determined in globulin the glycine, cystine, threonine, isoleucine, and methionine (Table 4), data for the content of which in this protein are lacking in the literature. The threonine content in these preparations varied from 1.96 to 2.80%, methionine from 1.03 to 17.3%, glycine from 10.63 to 13.10%, sum of leucine and isoleucine from 12.8 to 13.71%, sum of cystine and cysteine from 1.90 to 2.70%.

When we compare the results on amino acid content in all three protein fractions isolated from wheat grain proteins, gliadin, albumin, and globulin (Tables 2-4) we see that although the amino acid composition of all the proteins is the same, yet, in content of the individual amino acids gliadin differs greatly from albumin and globulin, while albumin and globulin differ from each other in less degree.

When we compare our data on the amino acid content with the data given in the literature, it becomes clear that in most cases there is a considerable difference. Thus, in gliadin preparations the glycine content from our data is 5.77-9.19%, and from the data of Patton [17] it is 0.47%; the content of serine from our data is 6.61-7.47% and from the data of Vickery [23] and Bishop [18] it is 0.10% and from the data of Rees [19], 4.38%; the valine content from our data is 2.16-2.80% and from the data of Osborn and Clapp [15], 0.20%; the content of glutamic acid from our data is 28.90-34.67%, and from the data of Cossel and Kutcher [16], 18.59%; the content of arginine from our data is 3.25-4.55%, and from the data of Hoffman and Gortner [20], 7.99%; the content of histidine according to our data is 2.50-3.54% and according to the data of Osborn and Leavenworth [21] and Osborn and Clapp [15], 0.6% and according to Blich [22], 6.20%.

We could give a number of similar examples for each of the amino acids, but these examples are sufficient to show that there is a great difference between our data and those of the literature as to the amino acid content in wheat proteins. We have also shown, above, differences in the results obtained by different authors.

The basic reason for such a wide variety of results is the use by the authors of different methods of obtaining

TABLE 5

Amino Acid Content in Proteins from Grains of Different Types of Wheat Grown under the Same Conditions (Leninakan Agricultural Station) In % of dry weight of protein

Amino acids	Albumin			Globulin			Gliadin		
	"Galgalos"	"Erinaseum"	Difference %	"Galgalos"	"Erinatseum"	Difference %*	"Galgalos"	"Erinatseum"	Difference, %*
Arginine	7,52	8,18	8,7	8,57	9,58	12,9	3,25	4,55	40,0
Histidine	6,57	5,36	24,4	4,81	6,28	30,6	3,08	3,54	14,9
Lysine	2,76	3,37	22,1	3,21	4,04	7,6	1,20	0,80	50.0
Tyrosine	3,00	4,85	61,1	3,23	4,82	49,2	1,35	1,39	2,9
Tryptophan	2,55	2,10	20,4	1,00	1,53	53,0	0,70	0,67	4,4
Phenylalanine	2,76	2,50	10,4	4,42	3,47	27,3	2,27	3,74	64,7
Proline	1,64	2,00	18,0	2,00	2,50	25,0	12,30	13,00	5,6
Cystine + cysteine	2,40	2,10	14,2	2,30	2,70	17,3	1,90	2,90	52,6
Methionine	0,68	0,66	3,0	1,23	1,73	40,6	0,30	0,37	23,3
Serine	6,77	7,73	14,1	7,14	9,35	30,9	7,34	7,61	3,6
Threonine	2,10	2,61	24,2	2,00	2,80	40,0	1,22	0,94	29,6
Leucine + isoleucine	13,21	14,35	8,7	13,71	13,08	4,8	11,50	12,78	11,1
Valine	4,83	5,21	5,2	6,00	4,26	40,8	2,16	2,65	22,7
Glutamic acid	13,50	15,24	12,8	14,00	13,11	6,7	31,18	28,90	11,3
Aspartic acid	6,46	9,98	54,4	7,81	9,57	21,2	1,98	1,58	25,3
Glycine	13,46	9,71	38,6	13,10	12,21	7,2	5,77	9,19	59,2
Alanine	2,48	3,96	59,6	4,00	4,67	16,7	2,61	3,69	49,4

*In calculating the difference in amino acid content, the minimum amount was taken as 100%.

the proteins and various ways of determining their amino acid composition. Also, protein preparations have often been isolated from different anatomical parts of the grain (from the endosperm, etc). Often we are not even told the object of study (variety of wheat, type of flour, etc) from which the protein preparation was obtained.

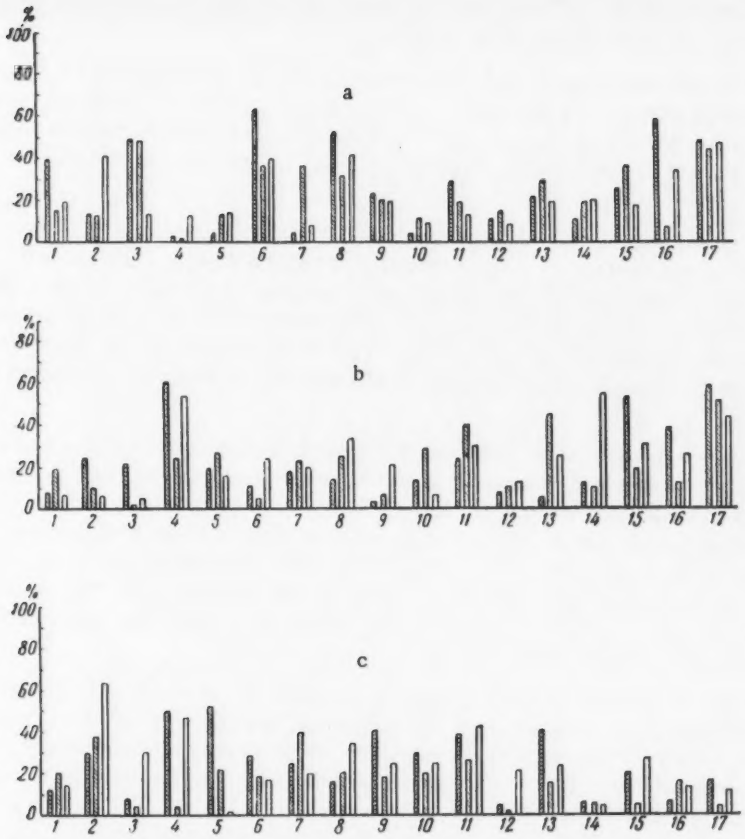
There are also in the literature a number of other reports of the amino acid composition of wheat proteins which agree closely with ours.

Thus, for example, in gliadin preparations the valine content is 2.16-2.80% from our data, and 3.3% from the data of Vickery [23]; the glutamic acid content is 28.90-34.67% from our data and 36.5% from the data of Abderhalden and Samuely [24] and 37.3% from the data of Osborn and Clapp [15]; the tryptophan content is 0.67-0.80% from our data and 0.7% from the data of Jones et al [25] and 0.6% from the data of Kraus [26]. Analogous examples can also be given for albumin and globulin.

However, in the case of agreement of these data we do not consider it possible to speak of the validity of this agreement, because of the differences in conditions of preparation and conducting the analyses.

We also studied the effect of a complex of factors in the external environment and of the biological characteristics of the types of wheat on the amino acid content in these proteins (Tables 2, 4). The results given in these tables show that the content of the individual amino acids in wheat grain proteins varies quite considerably depending on the growing conditions of the plant. Thus, for example, in gliadin preparations (Table 2) from "Galgalos" grain grown under different conditions the variation for lysine is 50.0%, for histidine, 13.2%, for phenylalanine, 37.4%, for threonine, 19.6%, etc. In the "Érinatseum" variety also grown under different conditions the variation for histidine is 40.0%, for lysine, 14.2%, for alanine, 47.0%, for valine, 19.9%, for phenylalanine, 40.4%, etc. Analogous examples occur for albumin and globulin preparations (Tables 3 and 4).

Besides these very marked changes there are changes in the content of some amino acids which do not exceed the experimental error. Thus, for example, the variation in lysine content in albumin from the "Galgalos" type (Table 3) is 0.3%, of phenylalanine, 5.4%, and of lysine, 4.8%. Analogous cases for a number of amino acids (tyrosine, alanine, glutamic acid, etc) can be seen in Tables 2 and 4. These examples indicate that the



Effect of conditions of growth and biological characteristics of the variety on content of individual amino acids in gliadin (a), albumin (b), and globulin (c) in wheat grain. Double crosshatched columns) difference (in %) in content of individual amino acids in wheat proteins of "Galgalos" and "Érinatseum" varieties grown under the same conditions. Crosshatched column ("Galgalos" variety) and white columns ("Érinatseum" variety) variation (in %) in amino acid content of proteins for wheat grown under different conditions: 1) arginine; 2) histidine; 3) lysine; 4) tyrosine; 5) tryptophan; 6) phenylalanine; 7) proline; 8) cystine + cysteine; 9) methionine; 10) serine; 11) threonine; 12) leucine + isoleucine; 13) valine; 14) glutamic acid; 15) aspartic acid; 16) glycine; 17) alanine.

content of not all the amino acids in wheat proteins is changed with changes in growth conditions of the plants. The content of one amino acid may change greatly and of another, very slightly.

We also studied the amino acid content in the proteins depending on characteristics of the types of wheat. In Table 5 we give comparative data on amino acid content in protein fractions isolated from the grain of different types of wheat grown under the same conditions. The content in the proteins (albumins, globulins, and gliadin) of the individual amino acids depending on the biological characteristics of the type of wheat varied considerably.

Thus, for example, in albumin preparations isolated from these types of wheat the difference for aspartic acid is 54.4%, for glycine, 38.6%, for alanine, 59.6%, for histidine, 24.4%, for tyrosine, 61.1%. In the globulin preparations the difference for tryptophan is 53.0%, for the sum of cystine and cysteine, 40.0%, for valine, 40.8%, for serine, 30.9%, etc. In gliadin preparations, as in albumin and globulin, the content of a number of amino

acids (lysine, phenylalanine, alanine, histidine, sum of cystine and cysteine, etc) shows a rather great change depending on the biological characteristics of the variety.

It is also clear that the differences in content of valine (5.2%), and methionine (3.0%) in albumin; tyrosine (2.9%), serine (3.6%), tryptophan (4.4%), and proline (5.6%) in gliadin isolated from different types of wheat, do not exceed the errors of the method of determining the amino acids. This indicates that the amount of these amino acids as contrased with the above amino acids changes very slightly with the differences in variety of wheat.

The content of the same amino acid in different protein fractions depending on biological characteristics of the variety does not change in the same way. As the data of Table 5 show, the difference for tyrosine between preparations of albumin isolated from different types of grain, but grown under the same conditions is 61.1%, and for globulin and gliadin it is 49.2% and 2.9% respectively. The same result is obtained in comparing the data obtained for valine, lysine, tryptophan, and other amino acids. Thus, we can conclude that the content of the different amino acids in wheat grain proteins can vary in different degrees depending on biological characteristics of the type.

The question arises as to which of the factors studied (growth conditions or biological characteristics of the variety) has the decisive effect on the amino acid content in the wheat proteins. On the basis of the data which we have obtained (Tables 2-5) we have prepared the diagrams (figure) which permit comparison of the effect of biological characteristics of the variety with growth conditions on amino acid content in gliadin, albumin, and globulin in the grains of the different varieties of wheat. Neither of these factors shows a predominating effect on the change in amino acid content in the proteins studied. Also, from the figure and from Tables 2-4 we see that the amino acid content in the grain proteins of the same variety of wheat grown under different conditions does not change in the same way.

Thus, for example, the content of arginine, tyrosine, phenylalanine, glutamic acid, in albumin; histidine, lysine, tyrosine, tryptophan, in globulin; arginine, lysine, tyrosine, and glycine in gliadin in grain of the varieties "Galgalos" and "Érinatseum" depending on growth conditions change entirely differently. If the content of these amino acids in the proteins of one variety of wheat varies greatly, yet in the same proteins of another variety the change may be comparatively small.

This indicates that the reaction of the organism to the conditions of the external environment depends on the biological characteristics of the variety in the processes of ontogenesis, the culminating point of which is the formation of the seed, and this determines to a considerable degree the character and extent of the change of one or another amino acid in the wheat protein. Therefore, in evolving new varieties of wheat with desirable qualities it is especially important to take account of the biological characteristics of the variety along with the growth conditions. Only then can we get positive results in developing new, high quality varieties of wheat.

SUMMARY

We have studied the dependence of amino acid composition of gliadin, albumin, and globulin in wheat grains on the biological characteristics and conditions of growth of the plant.

Using the method of paper chromatography, we have identified in these proteins, with quantitative determinations, the following amino acids: arginine, histidine, lysine, tyrosine, tryptophan, phenylalanine, proline, cystine, cysteine, methionine, serine, threonine, leucine, isoleucine, valine, glutamic acid, aspartic acid, and alanine. The results show that these proteins do not differ from each other in qualitative amino acid composition. However, in their content of individual amino acids, gliadin, albumin, and globulin of wheat differ considerably from each other.

Glycine, isoleucine, threonine, cystine, methionine in globulin, and serine, threonine, isoleucine, cystine, and methionine in albumin of wheat grains have been determined for the first time.

We have shown that both the conditions of growth and the biological characteristics of the wheat varieties have a very great effect on the content of the individual amino acids in these proteins. The changes in amount of individual amino acids depending on these factors can reach 50-60%. The content of the same amino acid in different proteins or in preparations of the same protein does not change in the same way depending on growth conditions and biological characteristics of the variety of wheat.

We have also shown that the growth conditions and biological characteristics of the varieties are equally important factors in the change in amino acid content of wheat proteins. Therefore, in developing new varieties of wheat it is especially important to consider equally the growth conditions and the biological characteristics of the varieties.

LITERATURE CITED

[1] R. Block and D. Bolling, Amino Acid Composition of Proteins and Food Products,(Izd. Inostran. Lit. Moscow, 1949).*

[2] F. A. Csonka, J. Biol. Chem. 118, 147 (1937).

[3] F. A. Csonka, Cereal Chem. 14, 397 (1937).

[4] B. S. Miller, J. Y. Seiffe, J. A. Shellenberger and A. D. Miller, Cereal Chem. 27, 96 (1950).

[5] A. I. Taranova, Biokhimiya 16, 239 (1951).

[6] A. I. Taranova, Amino Acid Composition of Wheat Proteins Depending on Variety, Place of Growth, and Grist. Dissertation, Moscow (1952).

[7] P. N. Kizima, Selection and Seed Production in Field Culture, No. 5, 8 (1951).

[8] G. Kh. Agadzhanyan, S. I. Khrimlyan, A. A. Kocharyan and A. B. Bagdasaryan, Agricultural Zones of the Armenian SSR. Izd. Glav. Uprav. S-kh. Nauk M-va S. Kh. ArmSSR, Erevan (1956).

[9] J. W. Pence and A. H. Elder, Cereal Chem. 30, 275 (1953).

[10] V. L. Kretovich, Doklady VASKhNIL, No. 6/9, 313 (1937).

[11] L. S. Markosyan, Izv. AN ArmSSR 11, 217 (1958).

[12] M. J. Horn and D. B. Jones, J. Biol. Chem. 157, 1, 153 (1945).

[13] R. Archer, C. Fromageot and M. Jutiser, Biochim. et biophys. acta 15, 81 (1950).

[14] A. N. Belozerskii and N. I. Proskuryakov, Practical Handbook of Plant Biochemistry, Sov. Nauka, Moscow (1951).

[15] T. B. Osborn and E. H. Clapp, Amer. J. Physiol. 17, 231, (1906-07).

[16] A. Cossel and F. Z. Kutcher, Physiol. Chem. 41, 134 (1903).

[17] A. R. Patton, J. Biol. Chem. 108, 267 (1935).

[18] Bishop, cited by N. N. Ivanov, Biochemistry of Cultivated Plants, vol. 8, p. 42, Sel'khozgiz, Moscow (1948).

[19] M. H. Rees, Biochem. J. 40, 632 (1946).

[20] W. Hoffman and R. Gortner, Cereal Chem. 4, 221 (1927).

[21] T. B. Osborn and C. S. Leavenworth, Amer. J. Physiol. 23, 180 (1908-09).

[22] M. J. Blich, Industr. and Engng. Chem. 8, 138 (1916).

[23] H. B. Vickery, Federat Proc. 3, 110 (1944).

[24] E. Abdergalden and F. Samuely, Z. Physiol. Chem. 44, 276 (1905).

[25] D. B. Jones, C. A. Gersdorff and O. Moeller, J. Biol. Chem. 62, 183, (1924-25).

[26] J. Kraus-Bagins, J. Biol. Chem. 80, 549 (1928).

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HEXOKINASE ACTIVITY IN CELLS OF TISSUE CULTURES

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It was previously shown that even a short exposure of cells under conditions in vitro caused a sharp rise in hexokinase activity [1]. It was further established that all investigations of tissue explants were characterized by comparatively high activity of this enzyme and a corresponding level of glycolysis [2, 3].

The maintainance of a definite level of metabolism in cells of tissue cultures probably occurs because of the specific intracellular organization of the metabolic processes, since under conditions in vitro the cells lack any regulator effect which would assure a coordinating effect on metabolism in the organisms. Therefore the question of the structural localization of the enzymes in different cell fractions of tissue explants, especially of the hexokinases [4-6] is of great interest. Besides a study of this question, we have included in our work the solution of some methodological problems which occur unavoidably in determining enzymatic activity in different fractions of the cells of tissue cultures.

METHODS

As objects of study we used the primary culture of monkey kidney cortex (MK), continuous strain HEP-2 [7], and cells taken from monkey heart (MH) [8]. The conditions of culturing and also the process of calculating the cells was described earlier [1, 2]. For collecting the cells from a glass surface we usually used a 0.02% solution of versene or a 0.25% solution of trypsin; in some experiments the cells were scraped off of the glass surface with a nail brush. In all cases the cells were washed by the process decribed before [2].

The cells were broken up in a Potter-Elvehjem homogenizer with cooling in a 0.15 M KCl solution or a 0.25 M sucrose solution used in about the ratio 1:1. The degree of destruction of the cells was controlled under the microscope; as a rule in 50-70 sec the cell membrane was broken up. Fractionation of the cell contents was carried out on a Servall SS-1 centrifuge according to the method of Schneider [9] with some modification. The nuclei were precipitated in 10 min at 700 g and were twice washed at the same rate. The mitochondrial fraction was precipitated for 20 min at 10000 g and twice washed at the same rate. All manipulations of fractionation were carried out at 4°. The mitochondria were controlled under the phase contrast microscope and by succinic oxidase activity [10].

Hexokinase activity was determined by a somewhat different method than was used before [1, 2]. The period of incubation was shortened to 5 min, and the amount of ATP was increased 1.5 times compared to the concentration recommended by Long [11]. Under these conditions the linear relation between concentration of enzyme in the sample and amount of glucose which disappeared was more evident. As controls we used samples fixed before incubation and samples incubated without ATP. Hexokinase activity was expressed in micrograms of glucose which disappeared per 10^6 nuclei or per 1 mg nitrogen; the nitrogen was determined colorimetrically with Nessler reagent.

RESULTS AND DISCUSSION

The data on hexokinase activity in homogenates of MH cells, using the incubation mixture of Sols and Long [11, 12] showed that the percent decrease of glucose using both mixtures was practically the same (the

TABLE 1

Hexokinase Activity of MH, HEP-2, and MK with Different Processes for Removing the Cells from the Glass Surface. Decrease in glucose in $\mu g/10^6$ nuclei

Method of removing cells		
versene	trypsin	brush
MH culture		
12,4	13,0	9,3
10,6	8,3	9,1
7,2	8,3	7,9
HEP-2 culture		
13,0	—	9,0
14,6	13,1	—
11,9	12,4	12,7
7,5	8,7	—
MK culture		
9,4	9,5	13,0
10,9	12,0	12,8
11,2	10,3	—

TABLE 2

Succinic Oxidase Activity of Mitochondria Separated under Different Conditions. In μliters O_2/mg N/hour

Fraction and conditions of separation*	Succinic oxidase activity
Homogenate	49
Mitochondria (10000 g)	143
Mitochondria (20000 g)	125
Homogenate	43
Mitochondria (10000 g)	173
Mitochondria (20000 g)	143
Homogenate	49
Mitochondria (10000 g)	212

*Fractionation carried out in 0.25 M sucrose solution.

hexokinase activity of the homogenate was characterized by fall in glucose, in percent):

Sols mixture	Long mixture
23.4	24.4
41.0	47.0
23.4	20.6

In the following experiments the incubation medium was that recommended by Long [11] with some modifications as mentioned above.

In connection with the recent indication [13] that versene used to remove cells from the glass surface extracted a number of glycolytic enzymes from the cells, we compared the effect on hexokinase activity of the cells of various processes for removing them from glass (Table 1).

The results show clearly that under our experimental conditions the process for removing cells does not change the hexokinase activity of the homogenates (MK, MH, HEP-2). It follows that the data on hexokinase activity obtained in this work with NH and HEP-2 cells are somewhat higher than in the previous contribution [2]. It is possible that this is because of a change in method (time of incubation, amount of ATP in the sample); the possibility is also not excluded that during prolonged culturing there is also some change in metabolism. Recently, for example, Leslie [14] reported that the intensity of glycolysis in HEP-2 cells after two year keeping in vitro was somewhat increased.

In the next experiments we studied the conditions most favorable for separating mitochondria from MH cells. In Table 2 we give the results of experiments on determination of oxidase activity of mitochondria obtained at different gravities. As is known, a marked rise in succinic oxidase activity of the mitochondrial fraction compared to the starting homogenate is one of the criteria of the mitochondria. Under our conditions, as a rule, the activity of the succinic oxidase was higher in the precipitate obtained at 10000 g than in the precipitate collected at 20000 g. We should mention that in these experiments the separation of the mitochondria at different rates was carried out simultaneously on two centrifuges using the same homogenate. Thus, the time from the withdrawal of the cells from the nutrient medium until setting up the manometric experiments was the same.

In Table 3 we summarize our data on the distribution of nitrogen found in the samples of mitochondrial and nuclear fractions of homogenates of MH cells. These results are compared with the corresponding data obtained by us and from the literature for liver and tumor tissue [15, 16]. We must remark at once that our data on nitrogen distribution are definitely of an orienting character, since during homogenization, on the one hand it is hard to be sure that 100% of the cells are broken up, and on the other, there is probably also destruction of mitochondria. The nuclear fraction is thoroughly contaminated with cell fragments. However, even on the basis of these preliminary results we gain the impression that in their structural organization as well as in the comparatively low succinic oxidase activity (Table 2) the cells of tissue cultures have more in common with malignant cells than with normal ones.

TABLE 3

Distribution of Nitrogen in Different Fractions of MH, Liver, and Tumors [15, 16]. In % total nitrogen of the homogenate

Object studied	Fraction	
	Nuclei	Mitochondria
MH cells	31,0	10,2
	29,4	11,4
	38,0	10,5
	32,3	13,8
	40,0	11,0
Rat carcinoma [5]	37,2	8,3
Mouse hepatoma [16]	22,8	13,1
Mouse liver [16]	17,9	23,5
Rat liver *	22,0	26,0

*Our data.

TABLE 4

Hexokinase Activity of Separate Fractions Obtained at Different Rates Fall of glucose in μg/mg N

Rate	Experiment No.	Precipitate	Supernatant liquid
10 000×g	1	210	476
	2	141	197
	3	105	190
20 000×g	4	205	242
	5	176	305
	6	293	348
50 000×g	7	38	162
	8	142	336

Determination of the hexokinase activity of the different cell fractions from MH cells showed (see below) that the whole cellular organism had definite hexokinase activity. The accuracy of these results for the nucleus is still not clear since, as already mentioned above, the nuclear fraction was contaminated with cell fragments. A special study was made of the question whether the hexokinase activity observed in the mitochondria is actually present there, or perhaps the mitochondrial fraction had not been sufficiently washed from the supernatant liquid which has a high hexokinase activity. For a test of this question, we determined the hexokinase activity of mitochondria washed once and three times. We showed that the activity did not fall after washing three times (392 and 430) and so we can assume that the hexokinase activity is present in the mitochondrial fraction as such. It is interesting that, according to Straub [17], mitochondria isolated from ascitic cancer cells as opposed to mitochondria from the liver have a comparatively high hexokinase activity. This fact once more confirms the similarity of some of the characteristics of metabolism in malignant tissue and the cells of tissue cultures. The presence of hexokinase activity in the mitochondrial fraction of MH cells agrees with the data which we obtained on the ability of this fraction to undergo glycolysis [18].

We give below data which show that in different experiments the hexokinase activity of the homogenate and also of the separate fractions varies considerably (activity characterized by fall in glucose in μg/mg N):

Fraction I	Exper. 1	Exper. 2	Exper. 3
Homogenate	148	305	122
Nuclei	77	174	52
Mitochondria	143	210	105
Supernatant liquid	196	476	190

These variations are perhaps related to different states of the cells used for the experiment (number of degenerate forms, cells undergoing mitosis, etc). Also we should mention that morphologically the mitochondria of tissue culture cells show special lability [19].

It follows from the above results that the highest hexokinase activity is concentrated in the supernatant liquid. It is of great interest to explain whether it is impossible at rates greater than 10000 g to obtain a precipitate which contains the main part of the hexokinase activity of the supernatant liquid. It is known from the literature [20] that the main part of the hexokinase activity of a number of organs (for example, brain, heart) is concentrated in the fraction which comes down at 18000 g. At the same time, in the tissues of the pancreas, hexokinase activity cannot be concentrated even in the precipitate obtained as a result of centrifuging at 100000 g [21]. In Table 4 we give our results on the hexokinase activity of the precipitate and supernatant fraction obtained by centrifuging at different rates for 20 min. We can say that these results were obtained in different experiments and therefore a comparison is possible only between the activity of the precipitate and the supernatant liquid in each separate experiment.

On the basis of the figures in Table 4 it is evident that even at 50000 g most of the hexokinase activity does not concentrate in the precipitate, but remains in the supernatant liquid. The low hexokinase activity of the precipitate obtained at 50000 g (experiment 7) is probably explained by the fact that in this case the mitochondria were contaminated by considerable amounts of ballast protein. As to experiment 8, this is less indicative, since centrifuging in this case for technical reasons was carried out for only 10 min. Thus, on the basis of these data we can say that, although some of the hexokinase is concentrated in the mitochondria, most

of it is found in the supernatant liquid. The presence of hexokinase activity in the mitochondria and also their ability for glycolysis [18] indicates their high functional importance in the metabolism of the cells of tissue cultures.

SUMMARY

The hexokinase activity of the homogenates of three types of tissue cultures (a strain of cells obtained from monkey heart, a strain of cancer cells HEP-2, and cells of seven and ten day cultures of monkey kidney cortex) is not changed by three different methods of collecting the cells from the surface of glass (with solutions of trypsin or versene, or mechanically).

The mitochondria of the MH cell cultures separated at 10000 g have hexokinase activity, but a considerable part of the hexokinase remains in the supernatant fraction and is not concentrated in the precipitate obtained even at 50000 g.

The results are discussed from the viewpoint of the distribution of the enzyme in the different cell fractions and the generality of some characteristics of metabolism in malignant tissue and the cells of tissue cultures.

LITERATURE CITED

[1] V. N. Gershanovich, V. I. Agol, R. N. Étingof and S. G. Dzagurov, Biokhimiya 23, 453 (1958). *

[2] V. I. Agol, V. N. Gershanovich and R. N. Étingof, Biokhimiya 24, 101 (1959). *

[3] H. Eagle, S. Barban, M. Levy and H. O. Schulze, J. Biol. Chem. 233, 551 (1958).

[4] S. A. Neifakh and M. P. Fomina, Biokhimiya 23, 3 (1957). *

[5] W. S. Beck, J. Biol. Chem. 232, 251 (1958).

[6] V. S. Il'in, Ukr. Biokhim. Zh. 30, 911 (1958).

[7] H. W. Toolan, Cancer Res. 14, 660 (1954).

[8] J. E. Salk, Amer. J. Publ. Health 47, 1 (1957).

[9] W. C. Schneider, J. Biol. Chem. 176, 259 (1948).

[10] W. C. Schneider and V. Potter, J. Biol. Chem. 149, 217 (1943).

[11] C. Long, Biochem. J. 50, 407 (1952).

[12] A. Sols, Biochim. et biophys. acta 19, 144 (1956).

[13] D. Matzelt and J. Homann, Biochem. Z. 330, 245 (1958).

[14] J. Leslie, M. Jarnell and R. Crockett, IV Internat. Congr. Biochem. Brussel. p. 69 (1958).

[15] G. A. Le-Page and W. C. Schneider, J. Biol. Chem. 176, 1021 (1948).

[16] W. C. Schneider and G. H. Hogeboom, J. Nat. Cancer Inst. 10, 969 (1950).

[17] G. Acs, T. Garzo, G. Groos, J. Molnar, O. Stephaneck and F. B. Straub, Acta physiol. Hung. 8, 269 (1955).

[18] R. N. Étingof and A. A. Krichevskaya, Biokhimiya 25, (1960) (in press).

[19] J. Frederic and J. Chevremont, Arch. Biol. 63, 109 (1952).

[20] R. K. Crane and A. Sols, J. Biol. Chem. 203, 273 (1953).

[21] C. Villa Palasi, Rev. esp. fisiol. 13, 207 (1957).

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A GENERAL METHOD FOR MILD PARTIAL HYDROLYSIS OF SCLEROPROTEINS AND ITS APPLICATION TO SILK FIBROIN*

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The study of amino acid sequences in the peptide chains of scleroproteins and certain other insoluble proteins has considerably lagged behind corresponding studies of soluble proteins. This is apparently due to the entirely natural attention of investigators to the physiologically active proteins of importance to the organism – hormones and enzymes, and, in addition, to the difficulties of analyzing the amino acid sequences in peptide chains of insoluble proteins.

The most valuable results on structure of the peptide chain were obtained by enzyme hydrolysis, which has a quite marked specificity. The resultant large peptides could then be separated and each studied separately. Acid hydrolysis has played rather a supplementary role than a leading one in these studies, although possibly such an appraisal is not entirely accurate in all cases. Comparison of the macropeptides obtained by enzyme hydrolysis and the oligopeptides resulting from acid hydrolysis has made it possible to reconstruct the structure of the peptide chain in a number of water-soluble proteins.

The complete (keratins, silk fibroin) or partial (elastin) resistance of scleroproteins to the mammalian proteases usually employed has prevented effective use of this method. In order to carry it out it was found necessary to resort to conversion of these proteins to a soluble form by the action of oxidizing agents that rupture the disulfide bridges between chains [1, 2], or to reagents that rupture the hydrogen bonds [3-5].

Hydrolysis by dilute or concentrated acids with heating or in the cold generally gives a highly complex mixture of higher and lower peptides and amino acids, difficult to separate by any single method; the use of several methods is required, each based on different principles [6]. Hydrolysis by concentrated alkali in the cold or dilute alkali with heating results in racemization of many amino acids and, what is worse, to the destruction of some of them [6-8]. Because of these difficulties the group of insoluble proteins has been insufficiently studied in this respect.

In this paper is described the application to silk fibroin (a protein of interest from the point of view of the problem of biological systematization [9]) of a method for mild hydrolysis of scleroproteins with 0.15-0.4 N $Ba(OH)_2$ in the presence of Cu^{++}, which we worked out previously for elastin [10]. The first observations concerning silk fibroin were made by Traube and Harting [11], but were not further developed. Out conception of the mechanism of hydrolysis is as follows.

The action on proteins insoluble in water at room temperature of a saturated aqueous $Ba(OH)_2$ solution in the presence of Cu^{++} ions results in the binding of Cu^{++} to peptide groups and rupture of hydrogen bonds between polypeptide chains of the protein. Simultaneously the disulfide bridges are ruptured, as found for hair keratin. Then each peptide chain, losing contact with neighboring chains, becomes accessible to the hydrolytic action

*Part of this work was reported to the X All-Union Conference on Highmolecular Compounds (June 11-13), 1958, Moscow.

of $Ba(OH)_2$, which splits first of all the weakest peptide bonds. The thus formed peptides, chiefly of high molecular weight, go into solution as a copper ion complex (violet color of solution). In addition a small amount of low-molecular peptides is formed, also going into solution. This mild splitting was tested on a number of proteins. Rate of splitting depends on the nature of the protein and to a less extent on particle size.

Silk fibroin* went into solution in 1-1.5 days, wheat gliadin and pumpkin-seed globulin required up to 2 days (particle size was the same). Elastin of ox ligamentum nuchae (2-2.5 days), skin protein mixture (3 days), and keratin of human hair (about 4 days) dissolved particularly slowly. All the protein went completely into solution in all cases. The weakly alkaline hydrolyzates obtained can be fractionated by acidification into soluble and insoluble parts, and the former into a dialyzable part (low-molecular peptides) and undialyzable part (high-molecular peptides). The low-molecular peptide fraction can be further studied by two-dimensional paper chromatography.

The role of the copper ions in this type of hydrolysis is presumably due to its great complex-forming ability with respect to ammonia and its derivatives. The dense violet color of the solution plainly indicates the presence of such complexes. In order to determine if the formation of these complexes actually results in considerable acceleration of protein hydrolysis by $Ba(OH)_2$, control hydrolysis of silk and elastin was carried out under the same conditions but without copper ions.

The maintenance of silk or elastin during the three summer months (day temperature – 35± 5°, night – 20 ± 5°) in 0.38 N $Ba(OH)_2$ resulted in very mild hydrolysis of these proteins with splitting off of a small amount of amino acids and low-molecular peptides.

The hydrolysis of silk fibroin, fractionation of the hydrolyzate, and isolation of a number of individual peptides by paper chromatography was carried out as follows.

EXPERIMENTAL

5 g of silk fibroin (hybrid SANIISh É-1 × É-2), 2-2.5 g basic copper sulfate – $7CuO \cdot 2SO_3 \cdot 6H_2O$ – [12], and 100 ml 0.36-0.38 N $Ba(OH)_2$ were mixed with a magnetic stirrer in a tightly closed vessel to avoid access of CO_2 at 36-38° for 24-36 hours to complete solution of the protein. The dark violet solution was filtered on the suction filter from excess copper salt and $BaCO_3$; the filter was washed quantitatively with water. Filtrate and wash waters were brought to 200 ml and acidified with 20% CH_3COOH to pH 6.7-6.9 to maximal precipitation of the abundant blue precipitate (fraction A), which was filtered with suction and washed with water to a negative reaction for Ba^{++}. The dark blue filtrate and wash waters (fraction B) were quantitatively freed of barium with H_2SO_4 and of copper with H_2S and evaporated in a vacuum (pressure 10-20 mm; bath temperature ~45°) to a volume of 10-15 ml, resulting in precipitation from solution of a large amount of bright yellow precipitate. The precipitate and residual solution were transferred to a dialysis sac of 7% collodion and the inner volume brought to 50 ml (previous measuring), and then placed in a 200 ml measuring cylinder containing 100 ml distilled water.

After dialysis for 24 hours at 0-5° the cylinder contents were evaporated in a vacuum to a volume of 3-5 ml and dried in a vacuum dessicator over P_2O_5 (fraction B_1). Contents of the collodion sac were again dialyzed with a fresh portion of 100 ml of water and fraction B_2 was obtained. Fractions B_3 and B_4 were obtained in the same manner. Each fraction was dried as indicated above. The insoluble precipitate remaining in the sac (fraction B_5) comprised more than 80% of all the B fraction.

Fraction A was repeatedly extracted with 0.1% acetic acid as long as the extracts were a pure blue color. The combined blue extracts were quantitatively freed of copper with H_2S, and the filtrate evaporated in a vacuum to small volume and dried in a vacuum dessicator over P_2O_5 and NaOH (fraction A_1). The residual material was extracted with 0.5% acetic acid several times until the acetic acid was no longer colored a bluish-green. This extract was also freed of Cu with H_2S, evaporated, and dried in a vacuum over P_2O_5 and NaOH (fraction A_2). The remainder (fraction A_3) after extraction was gray and contained no Cu. This fraction was little studied; its amino acid composition was close to that of fractions A_1 and A_2.

Chromatography of hydrolyzates of fractions A_1 and A_2 revealed aspartic and glutamic acids, threonine, leucines, phenylalanine, and proline. They differed from fractions B_1 and B_2 in amino acid composition.

*L. I. Vasserfirer, G. A. Brodskaya and N. L. Sinyashin partcipitated in the work with this protein.

Fractions B_1 and B_2 in batches of 1.8-2.60 mg were subjected to two-dimensional chromatography on Leningrad paper No. 1 or 2. The first solvent was a water-saturated mixture of phenol: o-cresol (2:1); the second, a mixture of butanol: acetic acid: water (4:1:5), distance ~ 70-75 cm.

The presence of more than 20 spots in fractions B_1 and B_2 was established, i.e., more than 20 individual peptides, of which 10 have now been studied. Five of them had tyrosine at the N-terminal. One peptide contained five amino acids in the following ratio: Tyr_1-Val_1-Ser_2-Ala_6-$Glyc_0$. Other peptides were of simpler structure. The peptide indicated is apparently a prolongation of the peptide found previously [13]. The presence of valine distinguishes it from the peptide found by Smith et al [14].

With decrease in $Ba(OH)_2$ concentration during hydrolysis a much milder effect on the fibroin occurs and only a few peptide bonds are split, resulting in formation of particularly large peptides. For example, 0-15-0.20 N $Ba(OH)_2$ after 35 hours at 30° apparently split the fibroin only at a few points. After brining the filtered dark violet hydrolyzate to pH 6.9 (dense blue color) and removing the gray-blue precipitate (fraction A) formed, the filtrate (fraction B) gave spontaneously on standing an abundant precipitate. After two days the precipitate was collected, washed first with water and then 0.1% CH_3COOH to negative reaction for Ba^{++} and Cu^{++}, and dried (yield greater than 49%, based on fibroin taken). The precipitate was insoluble in water, dilute acids and bases, and organic solvents both in the cold and with heating. Chromatography of the entire hydrolyzate of this precipitate revealed the presence of only four amino acids: serine, glycine, alanine, and tyrosine.

RESULTS

The hydrolysis method described has the following positive features:

1. Protein splitting proceeds relatively rapidly (1-3 days).

2. The hydrolysis does not result in destruction of amino acids * and gives relatively large blocks of amino acids, affording an opportunity after separating them in pure form to study each protein separately (this facilitates the process of studying protein structure).

3. The fractionation procedure is very simple (it is possible to work rapidly and conduct procedures quantitatively).

4. The hydrolysis affects different peptide bonds than those affected by acid, thus giving peptides having a different structure than those formed in acid hydrolysis (this affords an opportunity to fill in the "gaps" in acid or enzyme hydrolysis).

5. The hydrolysis shows a certain specificity resembling enzyme hydrolysis; of ten relatively low-molecular peptides isolated from silk fibroin, five contained tyrosine as the terminal-N amino acid. Consequently the peptide bonds formed by the amino group of tyrosine were preferentially hydrolyzed by $Ba(OH)_2$ in the presence of Cu^{++}.

SUMMARY

A new method of mild partial hydrolysis is proposed for silk fibroin and other water-insoluble proteins, particularly sclero-proteins, with use of $Ba(OH)_2$ in the presence of Cu^{++}. High-molecular peptides are formed principally and a small amount of low-molecular peptides; they all go into solution as a Cu^{++} complex. After removal of Ba^{++} and Cu^{++} the oligopeptides can be fractionated.

The method is simple, has been found applicable to all the proteins tested (silk fibroin, elastin, hair keratin, wheat gliadin, pumpkin-seed globulin), and exhibits a certain specificity resembling that of proteases: the peptide bond formed by the tyrosine amino group is preferentially split.

A high-molecular peptide was isolated from fibroin (yield ~ 50%), consisting of only the four amino acids serine, glycine, alanine, and tyrosine.

*However, during keratin hydrolysis a small amount of dark precipitate (CuS) is found, indicating a splitting off of sulfur.

LITERATURE CITED

[1] G. Toennies and R. Homiller, J. Amer. Chem. Soc. 64, 3054 (1942).

[2] R. Consden and A. H. Gordon, Biochem. J. 43, p. X (1948); 46, 8 (1950).

[3] G. H. Garret and F. O. Howitt, J. Text. Inst. 32, p. T, 1 (1941).

[4] D. Coleman and F. O. Howitt, Proc. Roy. Soc. A190, 145 (1947).

[5] B. Drucker, R. Hainsworth and S. G. Smith, Shirley Inst. Mem. 26, 191 (1952-53).

[6] P. P. Denyuel'*, Collection: Proteins (translated from English), p. 101, Izd-vo inostr. lit. Moscow (1956).

[7] F. Sanger, Advances Protein Chem. 7, 1 (1952).

[8] T. Wieland and L. Wirth, Chem. Ber. 82, 468 (1949).

[9] K. G. Ioffe, Khim. nauka i prom-st', No. 5 (1959) (in press).

[10] K. G. Ioffe and V. M. Sorokin, Biokhimiya 19, 652 (1954).

[11] W. Traube and H. Harting, Chem. Ber. 68, 1405 (1935).

[12] G. Bomeman, Inorganic Preparations, p. 148, Goskhimtekhizdat, Leningrad (1953).

[13] K. G. Ioffe, Biokhimiya 19, 495 (1954).

[14] F. Lucas, J. T. B. Shaw and S. G. Smith, Biochem. J. 66, 468 (1957).

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SCIENTIFIC INFORMATION

CONFERENCE ON THE PROBLEM OF BIOCHEMICAL EFFECTS PRODUCED BY IONIZING RADIATION

O. Ya. Tereshchenko

A Conference on the Problem of the Biochemical Effects Produced by Ionizing Radiation took place March 27-28, 1959 in Moscow at the Institute of Biological and Medical Chemistry, Academy of Medical Sciences, USSR. The Conference was convened by the Committee on Medical Radiology, Ministry of Health, USSR.

In opening the Conference, Professor N. N. Demin briefly outlined the series of problems confronting present-day radiation biochemistry, stressing, in particular, the special importance of studies in the histochemical field, study of physicochemical and early biochemical changes, further thorough study of biological complexes, studies to unravel the biochemical aspects of regulatory mechanisms, appraisal of the role of biochemical changes in the development of disease, etc.

In the report of O. G. Zamyatina "Biosynthesis of Serum Albumin in Radiation Sickness in the Rat," it was shown that irradiation of animals in a dose of 1000 r inhibited serum albumin synthesis in the liver and in some cases accelerated its breakdown. However, these disturbances developed only during the height of the radiation sickness. In view of the absence of changes in the early period of illness, the author concludes that disturbance of protein biosynthesis is not a result of the primary action of ionizing radiation on this process.

L. I. Il'ina and R. V. Petrov reported on protein synthesis in organoids of tissue cells in radiation disease. The authors studied the simultaneous incorporation of two amino acids with radioactive label, Methionine-S_{35} and tyrosine-C_{14}, into proteins of various cell structures. They found that while the ratio methionine/tyrosine did not change appreciably after irradiation (1000 r) in proteins of nuclei, mitochondria, and hyaloplasm of liver and intestinal cells, this value decreased considerably in the mitochondria of both tissues (liver 5 times, intestine 2 times).

N. D. Petrova and L. T. Tutochkina studied protein metabolism in bone tissue and blood serum mucoid in disease due to strontium-90. In the bone tissue of the treated rats were found decreased yield of soluble proteins and "resistant protein," increased yield of collagenous gelatin, reduced activity of autolytic processes and ability of bone tissue extracts to split added protein substrate. Changes in the diaphysis and in epiphyseal-metaphyseal parts were of the same type; however, they were less marked and appeared later in the diaphysis. In parallel with these changes in bone tissue, and in correlation with their extent, the serum mucoid content increased in the blood. This parallelism indicates that the serum mucoid concentration can serve as a satisfactory index of the organism's condition. The authors also found that during the increase in serum mucoid concentration, both protein and carbohydrate components increase with a relative decrease in content of the hexosamine fraction.

It was further shown in the report of N. I. Elkina and I. A. Tseveleva "Mineral and Protein Metabolism in Bone Tissue in Disease Due to Plutonium," that the incorporation rate of calcium, phosphorus, and glycine into bone of the treated animals decreased considerably. In rats given plutonium-239 in the amount $1.89 \cdot 10^{-3}$ microcurie/g, the incorporation rate of Ca_{45} after a year was half the initial rate. Activity of alkaline phosphatase under these conditions decreased 25% in a year, 50% in a year and a half.

V. V. Konstantinova and R. E. Libinzon studied the effect of plutonium on content and renewal of nucleic acids in certain rabbit tissues. Animals were given intravenously a solution of the nitrate salt of Pu in the amount 7 microcuries/kg. The greatest decrease of RNA phosphorus in liver and spleen tissues (28 and 26%, respectively) was noted 3 months after administration. RNA phosphorus concentration in bone marrow tissue did not decrease.

The DNA phosphorus content in the irradiated animals was decreased in all organs studied, being 36-56% of the initial level. The specific activity of RNA in liver, spleen and bone marrow, and the specific activity of DNA in spleen and bone marrow were increased. Increase in depolymerase activity in bone marrow was found 6 months after plutonium administration: 70% for desoxyribonuclease, and 96 and 240%, respectively, for acid and alkaline ribonucleases.

T. N. Rysina gave a report on the subject "Content of Free Nucleotides, Nucleosides, Purine and Pyrimidine Bases in Certain Tissues of Normal and Irradiated Rabbits." The author found that under the influence of total irradiation (1000 r) the uridylic acid content was increased in liver, appendix, spleen, and bone marrow, and that cytidylic acid, absent in the normal, appeared in a number of cases; the uridine content decreased in spleen and bone marrow. Among purine derivatives the greatest changes were found in content of inosine (increase in liver and appendix), adenosine (increase in liver, decrease in spleen and bone marrow), adenylic acid (increase in liver), and hypoxanthine (increase in liver, decrease in spleen).

E. F. Romantsev and Z. I. Zhulanova studied the effect of irradiation and certain protective substances on elimination of desoxycytidine in urine. The authors showed that under the influence of irradiation in high dosage (130 r/sec) the elimination of desoxycytidine in urine increases in proportion to the dose (in the interval 390-910 r), thus confirming the data of their Czech colleagues (I. Parizek et al), obtained with a considerably lower dosage. The preventive agents, l-cysteine and β-mercaptoethylamine, appreciably normalized desoxycytidine elimination in urine of the irradiated rats. The authors consider that one aspect of the mechanism of action of these agents is their normalizing effect on DNA metabolism.

N. A. Mkrtumova, T. A. Fedorova and V. P. Fedotov presented information on uric acid and allantoin contents in urine and blood of animals treated with ionizing radiation. Under the action of γ- and α-rays the allantoin content in blood and urine increased, and uric acid decreased. At the height of the radiation sickness, retention of uric acid in the liver increased and retention of allantoin decreased.

E. M. Belyaeva and S. S. Vasileiskii reported results of an immunoelectrophoretic analysis of blood serum proteins in radiation sickness. It was found that one of the precipitation arcs in the albumin fraction disappears in irradiated rats.

V. D. Blokhina gave a report "Distribution of Lipides in Cytoplasm of Liver Cells in Acute Radiation Sickness." The author found that in rabbits irradiated with 1000 r a transient increase in lipide content of the hyaloplasm occurs, chiefly at the expense of the stably bound fraction. After a brief rise in content of bound lipides in the cytoplasmic organoids, a sharp decrease is subsequently (in 72 hours and later) observed. Content of free lipides in cell organoids was found to be increased both in early phases after treatment and at the height of the sickness.

A. S. Kainova studied the effect of ionizing radiation on phosphlipides (PhL) of the liver. In rats totally irradiated with γ-rays of Co_{60} in a dose of 1000 r, the earliest changes were found 24 hours after treatment; sphingomyelin and lecithin fractions increased (42 and 34%, respectively), and cephalin decreased (57%). The total activity of PhL metabolism (determined from P_{32} administration) 4 hours later exceeded the normal by 100%; subsequently (6 and 24 hours) it decreased somewhat but remained above normal. The dynamics of the metabolism activity of individual PhL fractions coincided in general with that for total PhL. An exception was the sphingomyelin fraction, whose metabolism activity was decreased after 4 hours, and then rose also. In animals irradiated with doses of 5000, 15,000 and 30,000 r the changes were of the same nature, but appeared 10-30 minutes after treatment. The author considers the increased activity of PhL metabolism in radiation disease to be related to the destruction of lipoprotein complexes observed under these conditions, resulting in the freed PhL fractions becoming more accessible to enzyme action.

K. V. Smirnov and V. A. Shaternikov studied "The Effect of External Ionizing Radiation on the Role of Liver and Intestine in Lipide Metabolism." Working on animals subjected to angiostomy the authors found that, in the fasting normal, the blood in passing through the intestinal wall loses part of its total lipides, neutral fats and phospholipids; the extent of unsaturation of fatty acids in these compounds was increased. In passing through the liver, on the contrary, the blood content of total lipides, neutral fats and phospholipides increased and the iodine number of these fractions decreased. After irradiation the behavior typical of the normal was sharply disturbed. Thus, two hours after irradiation and in the terminal period of acute radiation sickness the intestine, as a rule, gave off total lipides, neutral fats and phospholipides to the blood; the iodine number of lipides in

the terminal period generally decreased. On the third day after irradiation, as a result of passage of blood through the liver, its concentration of total lipides, neutral fats and phospholipides decreased and their iodine number increased; in the terminal period of radiation sickness, after passage of the blood through the liver, the content of lipides and its fractions increased and their iodine number decreased. In the remaining phases a clear picture could not be obtained.

L. I. Polikarpova and A. Ya. Shulyatikova studied the dynamics of certain indexes of carbohydrate metabolism in monkeys with acute radiation sickness. The animals were irradiated totally the x-rays in a dose of 700 r. Within 24 hours after irradiation a certain decrease in blood sugar was noted in 3 of the 15 monkeys studied; in the other animals it remained without substantial change. Lactic acid content decreased considerably at this time. Polysaccharide content in blood of 6 of 12 monkeys decreased in 24 hours 1.5-2 times through decrease in amount in the formed elements of the blood. After three days the hypoglycemic effects noted disappeared and the sugar content varied within normal limits. Lactic acid and polysaccharides remained at the level observed after 24 hours. In the preterminal period a marked hyperglycemia developed, increased lactic acid concentration occurred, and there were no uniform changes in polysaccharide content. Three days after irradiation the glycogen content decreased in the liver and increased in the muscles. In the preterminal period these changes were still more marked. In none of the phases studied were changes in glycogen of brain or heart muscle tissues noted.

G. I. Bezin studied blood gases after treatment of dogs with polonium. It was found that hypoxia of the circulatory type arises in the course of development of radiation sickness. Symptoms of hyoxic hypoxia (decrease in oxygen-saturation of arterial blood) were noted in some of the animals with a subacute course of the disease. All the dogs showed an increasing arterial hypocapnia, which the author is inclined to regard as a compensatory reaction of the organism in response to accumulation in tissues and blood of incompletely oxidized metabolic products. The increase in oxygen capacity indicates change in the functional properties of hemoglobin.

E. M. Kedrova studied changes in tissue sulfhydryl groups under the influence of total irradiation with x-rays. In addition to data on the dynamics of SH-group content in organ homogenates in the irradiated animals, the author has given analogous data for individual cell fractions – nuclei, mitochondria, microsomes, and hyaloplasm. Most sensitive to the action of x-rays were found to be the nuclear proteins, in both in vivo and in vitro experiments. The SH-groups of microsome proteins are very sensitive to irradiation in solution, but resistant when a preparation of isolated microsomes is irradiated. Changes in microsome proteins were found later with in vivo irradiation than in in vitro experiments. Least sensitive to ionizing radiation were proteins of microsomes and hyaloplasm. The author concludes that an absence of inactivation of SH-groups by irradiation cannot be inferred on the basis of an absence of decrease in their total number in tissues of irradiated animals.

V. N. Benevolenskii, A. I. Zhuravlev and M. A. Lomova studied the toxicity of irradiated and oxidized fat preparations. After irradiation of sunflower oil with γ-rays in a dose of $1.35 \cdot 10^6$ r, the naturally occurring process of peroxide formation is accelerated and later increase in acidity of the preparation is observed. A preparation containing a large amount of peroxides has the ability to decrease SH-group concentration in vitro, but has no biological activity. Fat, oxidized to the stage where increase in amount of free fatty acids occurs, on intraabdominal administration causes death of the treated animals.

Zh. A. Antakol'skaya studied the effect of hyperthyroidosis on survival and SH-group content in soluble proteins of rat liver after irradiation with x-rays. The author found that thyroidin treatment prior to irradiation results in earlier death of the animals and greater decrease in number of SH-groups in proteins than in experiments with irradiation alone.

M. A. Lomova showed in the report "Sulfhydryl Groups and Components of the Adenylic System of Macacus Rhesus in Acute Radiation Sickness" that total SH-group content in liver and brain tissues, and in some animals in the whole blood, decreased as the disease developed. The author found a certain increase in ATP content of liver tissue 6 hours after irradiation with subsequent return to the initial level.

N. N. Demin, N. V. Korneeva, K. V. Smirnov and V. A. Shaternikov studied the effect of ionizing radiation on acetylcholine (ACh) metabolism. Irradiation of animals (rabbits) with γ-rays of high power in more than lethal doses (5000-30,000 r) resulted in early (in 10 minutes) and considerable decrease in content of bound ACh in brain, with considerable increase in cholinesterase activity. The action of radiant energy in a dose of 1000 r produced in the first two days in rats a decreased content of free ACh in brain and liver; bound ACh in brain decreased only on the second day and rather increased in the liver.

In monkeys irraidated with a dose of 700 r the cholinesterase activity in liver, small intestine, and brain tissues was increased on the third day and decreased subsequently. The authors conclude that disturbance in ACh metabolism plays an appreciable role in the pathogenesis of radiation sickness.

L. I. Polikarpova studied ascorbic acid metabolism in acute radiation sickness. One hour after irradiating rabbits with a dose of 1000 r, decrease in content of ascorbic (AA) and desoxyascorbic (DAA) acids in the aorta wall was found with decrease in the ratio DAA : AA. After increasing somewhat at 6 hours, the AA concentration continued to fall, while the DAA level stabilized. In guinea pigs a sharp increase in the DAA : AA ratio (of 5 times) on the third-fifth days was typical. Recalling the role of ascorbic acid in maintaining the stability of intercellular substance, the observed changes might be thought to concern the mechanism of development of hemorrhagia.

The following participated in the discussion of the reports: N. A. Fedorov, D. A. Chetverikov, S. Ya. Kaplanskii, A. E. Braunshtein, A. V. Kotel'nikova, S. E. Manoilov, D. E. Grodzenskii, V. M. Rodionov, T. A. Fedorova, R. E. Libinzon, L. L. Il'ina, N. N. Demin, B. M. Graevskaya, S. R. Zubkova, N. E. Kuznetsova, G. A. Kritskii.

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V INTERNATIONAL BIOCHEMICAL CONGRESS (MOSCOW, USSR), AUGUST 10-16, 1961

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General Information

In accordance with the resolution of the General Assembly of the International Biochemical Union, the V International Biochemical Congress will convene in Moscow, August 10-16, 1961.

The All-Union Biochemical Society invites all persons working in a field of biochemistry to participate as members in this Congress. Each participant in the Congress is entitled to invite a member of his family as guest to the Congress.

Persons desiring to participate in the V International Biochemical Congress should fill out the enclosed registration forms (two copies) and send them to the Executive Committee of the Congress (USSR, Moscow V-71, Lenin Prospect, 33) by March 31, 1961 for form I, and by December 31, 1960 for form II.

Persons desiring to give reports or demonstrations to sections of the Congress should furnish a brief abstract of their report (not more than 200 words) in triplicate by December 31, 1960.

Membership dues of $13 for members of the Congress and $2 for its guests should be transmitted to the current account of the Congress, No. 380083 in Vneshtorgbank [Foreign Trade Bank] USSR by March 31, 1961. Membership dues are not returnable.

Members of the Congress registering by March 31, 1961 will receive a second circular containing detailed information on organization of the Congress, program of the Congress, pamphlet of abstracts of section reports, and two pamphlets of symposium reports and communications of their choice. Materials of other symposiums can be obtained on additional payment. After the Congress the texts of the two lectures given at plenary meetings of the Congress will also be sent out free of charge. Members of the Congress unable to attend in person will receive all materials by mail.

Scientific Program of the Congress

In accordance with the wishes of the General Assembly of the International Biochemical Union, the organizers of the Congress intend to concentrate attention on a limited group of the most essential and promising topics in present-day biochemistry, for which purpose there will be organized:

I. Lectures at plenary meetings
II. Symposiums
III. Section meetings with reports and demonstrations

I. At Plenary Meetings of the Congress two Lectures will be Given:

a) D. Green (USA)— Subcellular Structure and Enzyme Function.

b) F. Shorm (Czechoslovakia)— Protein Microstructure in Relation to Biological Function.

II. The Following Symposiums will be Organized:

1. Biopolymers, Their Molecular Structure and Biochemical Activity. Three-dimensional structure of the most important biopolymers— proteins and nucleic acids. Interrelation of molecular structure and biochemical properties of enzymes, hormones, viruses. Enzymatic synthesis of polymers and mechanism of reproduction. Molecular structure of contractile proteins. Structural chemistry of antigens and antibodies; physical and chemical methods of studying biopolymers.

2. Functional Biochemistry of Cell Structures. Localization of biochemical functions within the cell, change in enzyme properties dueing development. Interrelations of various intracellular structures in metabolism. Questions of the comparative biochemistry of intracellular structures.

3. Evolutionary Biochemistry. Various pathways of carbohydrate metabolism in relation to evolutionary development. Evolution of anaerobic metabolism. Origin and evolution of aerobiosis. Origin and evolution of autotrophism— chemosynthesis and photosynthesis. Evolution of nitrogen metabolism.

4. General Mechanisms of Action and of Specific Inhibition of Enzymes. Examination of chemism of enzyme reactions at the molecular level. Questions of structure of catalytically active centers of enzyme proteins, their blocking by specific reagents, interpretation of enzyme reactions on the basis of modern theories of structure and transformation of organic molecules. Mechanisms of interaction between enzymes and structural analogs of substrates and coenzymes (antimetabolites).

5. Phosphorylative and Nonphosphorylative Oxidation. General principles of mechanisms of phosphorylative and nonphosphorylative pathways of oxidation, coupled reactions and their relation to mitochondrial structure. Function of oxidation in energy metabolism (accumulation of energy in utilizable form and heat production) and in protein metabolism.

6. Mechanism of Photosynthesis. Nature of the primary photochemical process. Photochemistry and spectroscopy of pigments. Enzymatic steps in photosynthesis, photosynthetic phosphorylation. Biosynthesis of pigments and their state in the organism. Photosynthesis products and their relation to respiration and nitrogen metabolism. Bacterial photosynthesis.

7. Basic Mechanisms and Pathways of Biosynthesis. Contents of the symposium will be determined when final program arrangements are made.

8. Biochemical Basis of Food Technology. Biochemical principles in selecting agricultural plants. Biochemical basis of technology of raw materials of plant and animal origin. Biochemical basis of fermentation technology.

III. The Following Sections will be Organized:

1. Chemistry of Biologically Important Compounds.
2. Biochemistry of Proteins and Amino Acids.
3. Biochemistry of Nucleic Acids and Nucleoproteins.
4. Biochemistry of Viruses.
5. Enzymology.
6. Comparative Biochemistry.
7. Biochemistry of Antibiotics.
8. Industrial Biochemistry.
9. Cyto- and Histochemistry.
10. Biochemistry of Muscle.
11. Biochemistry of the Nervous System.
12. Vitamins and Nutrition.
13. Biochemistry of Hormones.
14. Biochemistry of Microorganisms.
15. Biochemistry of Plants.
16. Medical Biochemistry.
17. Biochemistry of Carbohydrates.
18. Biochemical Pharmacology.
19. Biochemical Genetics.
20. Immunochemistry.
21. Biochemistry of Malignant Tumors.
22. Biochemistry of Photosynthesis.
23. Respiration and Oxidation;
24. Action of Radiation on Biochemical Processes.
25. Biogeochemistry.
26. New Apparatus and Methods in Biochemistry.
27. Miscellaneous.

Each member of the Congress may be entitled to give one section report. Reports may be made by the author whose name stands in first place in the study presented. In addition, a member of the Congress may participate as co-author in two further reports. The Secretariat reserves the right in the event of shortage of time to read certain studies by title only.

Authors of reports may use any language; however, in order to facilitate information exchange it is desirable that authors use Russian, English or French.

Tables and figures for demonstration with the epidiascope or projector should be of standard dimensions.

Exhibits

It is proposed to organize during the V International Biochemical Congress an exhibit of scientific literature on biochemistry and related fields of knowledge, and also an exhibit of instruments and apparatus, reagents, and preparations employed in biochemical investigations.

Excursions and Program for Ladies

Excursions to points of interest in Moscow and its vicinity will be organized for participants in the Congress and members of their families. A detailed program of these will be supplied to Congress participants later.

Accomodations

Intourist will furnish participants of the Congress accomodations in the following classes:

Class "Lyuks" [de luxe] at a cost of 120 rubles ($30) per day. This price includes private room with bath for 1 person, 4 meals a la carte, passenger car and interpreter for 2-3 hours daily, transportation to and from station or airport in passenger car and porter service. If two are traveling in the de luxe class and staying in the same room, the cost is 108 rubles ($27) per day per person.

First class at a cost of 70 rubles ($17.50) per day. This price includes room with bath for 1 person, 3 meals from first-class menu, conveyance by bus, interpreter service on the basis of one interpreter per 20-25 persons. For two tourists staying in the same room the cost of accomodations for the Congress first class is 63 rubles ($15.75) per day per person.

Tourist class at a cost of 50 rubles ($12.50) per day. This price includes room with bath or shower for 2-3 persons, 3 meals daily from tourist-class menu, daily excursions in buses, and interpreter service on the basis of one interpreter per 20-25 persons.

Accomodations for students are offered at a cost of 17.20 rubles ($4.30) per day. This price includes accomodation in student dormitories, meals in the dining room, use of buses and interpreter service up to 3 hours per day.

Since Congress participants will arrive in the USSR and depart from it individually, arrival and departure transportation must be paid for in addition. This applies to Congress participants with first class, tourist class, or student tour accomodations. Cost of transportation to and from airport or station on arrival in the USSR and departure from the USSR by passenger car is 20 rubles ($5) per person, separately for arrival and for departure, with the proviso that this type of service be purchased abroad.

The cost of the classes indicated above does not include payment for personal expenses, such as barbershop services, theater or movie tickets, etc. These services are paid for in rubles with cash for actual expenses.

If any Congress participant on arrival in the USSR desires to obtain room or meals of a higher class, he may do so by paying an additional amount in rubles with cash for actual expenses.

Payment and arrangements for the trip, including application for visas, are made through tourist agencies— Intourist agents. A list of the agencies in different countries, recommended by Intourist to Congress participants for making arrangements for the trip, is enclosed.

Congress participants from countries of the People's Democracies, together with the procedure mentioned above of paying for the trip through an agency, may also make payment for accomodations in cash in rubles, after cashing a draft on the People's Bank of his country, i.e., without paying for a tour at an agency, but by paying for actual expenses.

Those persons coming from countries not having diplomatic relations with the USSR at the present time, or from countries where Intourist has no agents, are advised to apply to the USSR Embassy and also an agency in any country most convenient for these people, for instance in a country through which they will pass in transit.

Persons desiring to arrive in the USSR by automobile can come through Rumania, Poland or Finland. All details can be learned from the tourist agencies — Intourist agents.

K. U. LINDERSTRØM-LANG

Professor Kaj Ulrik Linderstrøm-Lang, eminent Danish biochemist, Doctor of Philosophical and Technical Sciences, foreign member of the Academy of Sciences USSR, died on May 25 in his sixty-third year. Member of the Royal Academy of Sciences and Academy of Technical Sciences in Copenhagen, he headed the universally famous Carlsberg Laboratory and was a member of the Danish National Research Council.

He has written about 100 scientific papers, dealing with the study of proteins, with histological investigations. Among them are "Studies on Caseins" (1925), "Studies on Proteins," "On the State and Stability of Colloidal Solutions," "Effect of Salts on Ionization of Egg Albumins" (1926), "Studies on Histochemistry of Enzymes" (1933), "Exchange of Phosphorus Atoms in Plants and Seeds" (1937), "Micromethods in Histochemistry" (1939), "Number of Peptide Bonds in Insulin" (1940). In recent years Linderstrøm-Lang has published such studies as "Exchange of Hydrogen Atoms in Insulin with Deuterium Atoms in Aqueous Solutions," "Exchange of Deuterium and Oxygen between Water and Other Substances," "Deuterium Exchange between Peptides and Water," etc.

The outstanding investigations of Linderstrøm-Lang have received worldwide recognition. He was a member of the Norwegian Academy of Sciences, the National Academy of Sciences in Washington, the American Philosophical Society, the French Biological Society, Honorary member of the Harvey Society, Belgian Chemical Society, and American Academy of Arts and Sciences. The biochemical community of the world elected him Secretary General and then President of the International Biochemical Union.

In 1958 Linderstrøm-Lang was elected a foreign member of the Academy of Sciences USSR. He was a member of the organization committee for the V International Biochemical Congress, which is to convene in Moscow in 1961.

Soviet scientists learned with deep sorrow of the death of Linderstrøm-Lang. Academician A. V. Topchiev, Vice-president of the Academy of Sciences USSR, in a telegram in the name of the Academy expressing sympathy to the Royal Academy of Sciences in Copenhagen on the loss sustained by Danish science, wrote "The Academy of Sciences USSR, of which Professor Linderstrøm-Lang was a foreign member, will always honor his bright memory."

Academician A. I. Oparin, Chairman of the National Committee of the National Committee of Soviet Biochemists and Director of the Institute of Biochemistry im. A. N. Bakh, sent telegrams expressing sympathy to the International Union of Biochemists and to the Carlsberg Laboratory.

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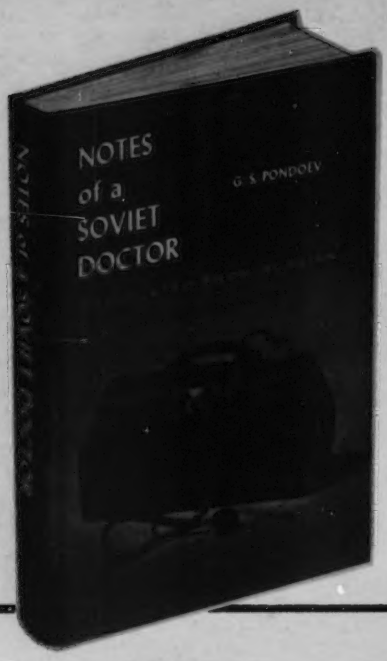

NOTES of a SOVIET DOCTOR

by

G. S. PONDOEV

Honored Physician of the Georgian SSR

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Professor D. G. Mamamtavrishvili,
Honored Scientist

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